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PHYSIOLOGICAL ZOÖLOGY

Volume XVII JULY 1944 Number 3

GENESIS OF SOMATIC MOTOR FUNCTION IN MAMMALIAN EMBRYOS: A SYNTHESIZING ARTICLE

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IN WRITING a synthesizing article on the genesis of motor functions I intend to consider the subject critically and mainly in terms of recent investigations. It is not my purpose to write a comprehensive review. I shall try to reconstruct the train of thoughts which have led to my present views and discuss the relationship of these to opinions of others. I hope this synthesis will help clarify the subject and that, when the time again arrives to renew studies of this character, workers will be able to proceed in greater harmony.

It is nearly twenty years since concerted efforts to study motility of mammalian embryos and genesis of nervous activity were begun. The investigations of Coghill, reported first in 1908 and in a long series of articles since that time, stimulated search for correlations between anatomical and physiological entities in development of the nervous system. It is desirable to consider briefly the history of the search for this knowledge, especially in regard to development of divergent views stemming largely from different technical approaches to the subject.

In the salamander *Amblystoma*, Coghill (1929*a*) demonstrated that somatic behavior starts from a fully integrated mass reaction or "total pattern," from which more discrete or simple movements differentiate by a process of "individuation" as growth proceeds. In this simple vertebrate more or less discrete movements are not the primary units of behavior; reflexes appear to originate from a background (in the Gestalt sense) of mass movement. No one has seriously questioned the soundness of these views, for Coghill (1929*a*) demonstrated the structural basis for his physiological observations in a series of painstaking investigations. He believed that he had discovered a general principle with wide, if not universal, application in vertebrates and quite naturally set about to test the validity of his total-pattern theory in other species. To this end he assigned Swenson the task of studying the intrauterine behavior of albino rat fetuses.

Swenson's observations failed to produce convincing evidence that genesis of behavior in mammals is like that in the salamander. On the other hand, his experiments were inadequate to show how the two forms differed. He (Swenson,

1929) described the early activities of the rat as simple movements, saying:

> Each simple movement makes its first appearance in a definite order in relation to other simple movements. Once a simple movement has appeared it persists in kind of movement but its quality changes with the development of the animal.

Swenson's (1926) results were never published in full.

It was about this time that Minkowski (1928) published a comprehensive summary of his studies on human fetuses. His observations (Minkowski, 1921, 1922) and those of Bolaffio and Artom (1924), as well as other scattered notes on human fetuses, convinced Coghill (1929*b*) that "the same law prevails in the development of behavior of human beings as that which has been observed in *Amblystoma*." It is unfortunate that Coghill's enthusiasm permitted him to bridge the chasm between *Amblystoma* and man with such confidence and without firsthand knowledge—in fact, in spite of the expressed doubts of his own student. It was not until several years later that the apparent confirmation mentioned in the next paragraph was obtained. In an article written in 1934 and published posthumously in 1943 Coghill discussed this gap between the salamander and man; but the inadequacy of his survey, in view of more recent studies, led the editor to delete much of it.

Physiological studies on albino rats were continued in Coghill's laboratory by Angulo, who had originally undertaken anatomical aspects of the problem with Swenson. In 1932 Angulo described results which appeared to demonstrate that behavior in this mammal has its genesis in mass activity patterns like those of *Amblystoma*. About this time, two investigations (Windle and Griffin, 1931; Coronios, 1933) on the cat appeared to support the Coghill theory. Griffin and I reported that "movements of embryos and young fetuses are to a large degree massive and generalized" and that "with a few exceptions there seems to be a gradual evolution or individuation of the more discrete unit reflexes from the total patterns"; however, we added the further observation that some "reflex movements seem to make their appearance with little or no relation to the primitive behavioral background." At this stage in the search for origins of behavior, investigators found themselves in agreement, substantially.

Nearly all first-experiments of investigators in behavioral development of mammals were conducted under unphysiologic and abnormal conditions—indeed, usually on deeply asphyxiated and dying fetuses. So little was known of intrauterine physiology that observers were insufficiently concerned about their ability to control experiments. Misconceptions of fundamental principles were commonly held. For example, most anatomists and physiologists believed that the fetus normally develops in a state approaching anoxia, for the venal caval blood stream appeared to them to mix in the fetal heart (Windle and Becker, 1940*a*).

Although it was well known that narcotic and anesthetic drugs depress or even abolish adult reflex activity, some workers did not hesitate to draw conclusions regarding embryologic development of somatic movements from experiments in which these agents were employed. For the most part, however, investigators did appreciate the deleterious effects of general anesthesia and avoided it. But this sometimes led to a false security, with a feeling that we could violate other physiologic principles with impunity if we just avoided anesthesia.

Minkowski (1928) often used local anesthesia for his human patients; but the fetuses were removed from the uterus before actual study was made, and there can be no doubt that asphyxia prevailed. The same criticism applies to most other studies in man. The rat experiments of Swenson (1926) and Angulo (1932) were carried out under ether anesthesia of varying depth, and the first ones of Barcroft and Barron (1936; see also Barcroft *et al.*, 1937) were done under urethane. It is interesting to note in Swenson's protocols that, whenever ether ceased to be effectively administered, unit reactions were more evident. Investigators using the cat and guinea pig (Windle and Griffin, 1931; Coronios, 1933; Carmichael, 1934) avoided anesthesia during experiments by previously sectioning the spinal cord or decerebrating the pregnant subjects. However, in our and probably other early studies delay after incising the uterus usually allowed anoxia to develop before observations were well begun. The use of techniques of decerebration, cord section, local and spinal anesthesia, together with speed of observation and careful handling of the uterus, ultimately led to observation of a type of fetal behavior which is unlike that occurring in *Amblystoma* and does not fit into the total-pattern concept. One must evaluate much that has been written about behavioral development critically, always bearing in mind the methods employed in the experiment. Use of great numbers of specimens and averaging of data are not substitutes for judicious planning of experiments or critical physiologic observations.

Significant species differences appear in the course of development of somatic activity, but in early stages of all that I have had occasion to examine personally (rat, guinea pig, sheep, cat, and man) there appear to be remarkable similarities. A certain amount of generalization would seem to be permissible.

The first somatic movements can be elicited by adequate stimulation shortly before the time at which external morphologic characteristics of the species become recognizable, i.e., before the period of the fetus is entered upon. However, we have no proof that embryos or young fetuses perform any movements *in utero* spontaneously. Not until approximately 14 weeks can intrauterine movements be detected in the intact human being, and the mother cannot feel them with certainty until about 17 weeks. How much earlier they normally begin we do not know. Quiescence *in utero* is an apparent fact in the early stages. When in an experimental animal the abdomen is opened and the gravid uterus is exposed without being handled, the fetuses within it appear singularly quiet. Other investigators have reported similar observations. In the sheep, an especially suitable animal for the purpose, Barron (1941) found, by transillumination, that as long as the uterus was unmolested the embryo was inactive. Manipulation during delivery led to activity. In the cat, changing uterine tonus and impairment of oxygenation of the fetal blood leads to activity in otherwise inactive fetuses.

Striated muscles develop before spontaneous activity can be observed. In fact, before reflex movements can be elicited in embryos, it is possible to induce movements by stimulating electrically directly over developing muscle masses. Contractions of embryonic muscles induced in this way have certain characteristics which distinguish them from true reflex movements. Chief of these is that each adequate stimulus produces a contraction; there is no interval of apparent fatigue during which the muscle is refractory, as there is in the case of a reflex. One movement after another can be obtained. Another characteristic is that contraction is localized in a small region immediately around the point stimulated. Consequently, movements in several planes can be produced by shifting the position of the stimulus. Finally, the embryonic muscle tissue retains a high degree of excitability, irrespective of changing metabolic conditions. Excitability is not nearly as quickly destroyed by asphyxiation as is excitability of the reflex arc. Specimens which have been allowed to bleed until white, which have cooled to room temperature, and in which the heart has practically ceased to beat still respond to stimulation of the skeletal musculature.

TABLE 1

SPECIES DIFFERENCES IN AGE AND SIZE OF SPECIMENS AT INCEPTION OF SOMATIC MOTILITY

Species	Gestation	First Movements (Days)	Size (Mm.)
Rat........	21 days	15–16	12–13
Guinea pig...	67–69 days	25–26	17–18
Cat.........	65–67 days	23–24	13–14
Sheep.......	21 weeks	33–34	24–25
Man........	38 weeks	54–60	22–24

Straus and Weddell (1940) have studied contractions of skeletal muscles in living rat fetuses produced by electrical stimulation in the forelimb region. The movements they obtained "exhibited high threshold, relatively long duration, tendency toward rapid fatigue, and long time factor (suggestive of both long latent period and long chronaxie of the muscles)." Fetal muscle does fatigue rapidly, in comparison with adult muscle; but, when one compares fetal muscle contractions with fetal reflexes, one finds that the muscle responds repeatedly to stimulation, while the early reflex is of a fleeting nature.

Straus and Weddell doubt the advisability of assuming a myogenic phase of development in which contractions occur without nervous function, and they believe that the evidence indicates potentially effective neuromuscular (nonsynaptic) transmission on the efferent side at 16 days in the rat. There is no doubt that growing nerve fibers are present in the mesenchyme at the time muscle fibers start to differentiate, but it is not proved that their presence is necessary for contraction of the new muscle. We know that chick skeletal muscle cells can contract in tissue culture in the absence of nerve fibers (Lewis, 1920).

Early reflex movements have been considered in a number of articles from this and other laboratories (Windle, O'Donnell, and Glasshagle, 1933; Windle, Orr, and Minear, 1934; Windle, Minear, Austin, and Orr, 1935; Carmichael, 1934; Bridgman and Carmichael, 1935; Barcroft, Barron, and Windle, 1936; Fitzgerald and Windle, 1942). About 1 day after it was possible to obtain muscle contraction in mammalian embryos for the first time, true reflex movements were elicited. The earliest responses of this type were movements of the forelimb or movements of the head. In the cat embryo the former type of reflex was present in some embryos which gave no head response. When head movement and forelimb movement were both present in the same specimen, there appeared to be no close relationship between the two; one or the other could be elicited independently. As development proceeded, it was possible to obtain similar movements in other regions.

There has been some question regarding the nature of these early movements. Some investigators have been unwilling to concede that they are reflexes, holding that they may as well be movements following conduction of impulses from the central nervous system to the muscle over motor nerve fibers but not involving sensory neurons and therefore not constituting true reflexes. The movements in question can be obtained from embryos in a number of ways. They do not occur spontaneously but follow external stimuli, which must be adequate.

When conditions as nearly normal as possible prevail in an experiment and the uterus is opened quickly to display the embryo within its amnion and still attached to the uterine wall by its placenta, movements can be obtained readily by tapping upon the amnionic sac with a blunt instrument or simply by imposing pressure upon the sac. There may follow a quick outward and backward jerk of the forelimb. A similar movement can be obtained by passing a sharp needle through the amnion and flipping the limb gently with it. Once in a while it is possible to obtain a similar response by touching the tip of the forelimb with a hair, but usually stronger stimuli are necessary. This is especially true in the early stages of development. These and other observations suggest that we are dealing with muscle-stretch reflexes in which a proprioceptive afferent neuron is involved.

Another somatic movement which makes its appearance almost as early as that of the forelimb is an extension of the head backward or to one side when the nose or mouth region is touched or stimulated with a faradic current. Because this movement is often a contralateral one and can be obtained more constantly with a "tactile" stimulus than can the limb response, all observers believe it to be an exteroceptive type of reflex.

The movement of the head in response to external stimulation is more resistant to changing physiologic conditions than is that of the forelimb. The forelimb movement is more easily abolished by anoxia than the movement of the head. This may be one of the reasons why some investigators have failed to obtain the movement of the forelimb while readily observing that of the head. Another reason is that inadequate stimuli have sometimes been employed. Hooker (1936, 1939) has taken precaution to use light tactile hairs for stimulating human fetuses after operation. His failure to observe the type of response seen in the forelimb of other species is explainable partly on this basis. Except in the earliest phase of anoxemia, a forelimb reflex cannot be obtained in small fetuses with tactile stimuli. If the forelimb reflexes are muscle-stretch reflexes, one should not expect to elicit them by tactile stimulation.

The early reflex movements of embryos have characteristics which serve to differentiate them from movements induced by direct stimulation of muscles. In case of the forelimb movement, it was often observed that the reflex could be obtained only once, although later the limb moved repeatedly when stimulated with faradic shocks directly over the muscle masses. At best, the forelimb movements were obtainable for only a few moments, while physiologic conditions were at their best. When more than one reflex of the forelimb was elicited from an embryo, it was invariably found that the second movement could not be obtained until a brief interval had elapsed after the first. There was a period of time during which the mechanism for the response was fatigued.

One of the most striking differences between the direct response to stimulating muscle and the early reflex of the forelimb was the stereotyped nature of the latter. The reflex was always a movement of the same type, and it occurred in the same direction, whereas the movement in response to direct stimulation of the fetal muscle was readily molded by the position of the stimulus. The limb could be caused to move backward or forward, depending upon the placement of the stimulating agent.

Although asphyxiation quickly abolished reflex movements of the forelimb, it usually changed the appearance of the head reflexes before they were abolished. For example, certain movements contralaterally became homolateral movements. More will be said about this reversal later.

Barron (Barcroft and Barron, 1937*a*) attempted to decide whether the early limb movements are reflexes by dissecting peripheral nerves of sheep fetuses and then stimulating them. The fetuses were considerably more advanced in development than those exhibiting the early forelimb reflexes in question. Nevertheless, he was unable to obtain reflex activity after applying faradic shocks to the central ends of dissected nerves, until from 44 to 47 days of gestation; he believed this to be evidence against the reflex nature of the early movements. The objection to these experiments is that, by the time a dissection can be completed, the central mechanism of any reflex, which may have been elicitable normally, will have suffered deleterious effects of prolonged exposure, asphyxiation, and trauma.

In the cat fetus, after reflexes are no longer obtainable one can insert an electrode into the spinal cord, stimulate, and obtain movements of the limb. A forward movement results from stimulating in the rostral part of the cervical enlargement, whereas a caudal movement of the limb results from stimulating the caudal part of the cervical enlargement. When a stimulus is applied in this manner to the thoracic or lumbar portion of the spinal cord, no movement follows if the embryos have been exposed for some time and are asphyxiated. However, if the embryos are in good physiologic condition and are receiving adequately oxygenated blood, as determined by the color of the umbilical veins, then electric stimuli placed in the spinal cord or even just over the spinal cord in the lower thoracic regions often induce reflex-like movements of the forelimbs. This is sound evidence of synaptic transmission, which is the very basis for reflex action.

In other experiments (Windle, 1937) on cat embryos in which sensory nerves had grown out distally into the limb beyond the region in which muscle cells had differentiated, it was possible to stimulate these nerve fibers electrically without stimulating the more proximal muscle masses. When this was done, but only during the time normal physiologic conditions prevailed in the embryo, reflex movements of the forelimb, like those obtained by pressure or by bending the limb, resulted. Subsequent anatomical studies in the same specimens revealed that structural components of reflex arcs were indeed present in these embryos.

Angulo (1935) attempted to determine the nature of the early movements of rat fetuses by experiments in which curare was injected. He reported that injection of 0.05 cc. of a 1 per cent solution of the drug abolished reflexes in 3 minutes but left fetal muscles responsive to direct stimulation. Here, again, it is possible that exposure of small fetuses for the time necessary for the experiments would be such that no reflex movements should be expected. Asphyxiation may well have been the cause of loss of reflexes. In respect to the time of action of the drug, Angulo's results are quite different from those obtained in guinea pigs by Preyer (1937). The latter found that a dosage adequate to curarize an adult guinea pig in 10 minutes failed to affect the fetuses when injected into them *in utero* until 52 minutes had elapsed. In fact, the drug passed through the placental barrier, from the fetal to the maternal side, and killed the mother before affecting the fetus.

Considerable anatomical evidence supports the view that certain elicited movements of embryos and young fetuses are reflexes. After performing experiments in this laboratory on embryos of the cat and other species of animals, nearly all specimens were preserved and stained by the Ranson pyridine-silver technique. Several reports on structural development of reflex arcs have been made (Windle, 1934; Windle and Baxter, 1936; Windle and Fitzgerald, 1937; Scharpenberg and Windle, 1938).

All nervous elements essential for reflex action are present in spinal cords of mammalian embryos shortly before the first reflex movements can be elicited by stimulation. However, the intrinsic synaptic connection between the primary afferent neuron and the primary efferent neuron are incomplete. One finds afferent nerve fibers running in the peripheral nerves to the tissues immediately beneath the epithelium of the forelimb and to deeper structures. The central branches of these neurons constitute the dorsal roots and dorsal funiculi of the spinal cord. Motor fibers arise from well-developed anterior horn cells in the spinal cord and pass through the ventral roots to the muscle masses of the forelimb and trunk. These nerve fibers can be seen to end in simple terminations upon the young muscle fibers. Within the spinal cord there are well-developed commissural and associational neurons whose cell bodies lie in the dorsal part of the gray matter. The commissural fibers are more numerous in the region just beneath the dorsomedial border of the dorsal funiculus, and association neurons are more numerous near the ventrolateral portion of this funiculus. The commissural fibers build the opposite ventral funiculus and come into close relation with motor neurons which supply the trunk muscles. Association fibers course into the lateral funiculus of the same side. They are very closely related positionally to the dendrons of the motor cells supplying the forelimb.

Before forelimb movements can be elicited reflexly, there appears to be no close relationship between the afferent neuron and the interneuron in the region of the dorsal funiculus. A few sensory dorsal root collaterals are present, but in no species examined (cat, rat, sheep, and man) do these nerve fibers extend very far into the region of the gray matter. A correlation can be drawn between the increase in number of collaterals and the occurrence of reflexes. Reflex arcs are completed by the ingrowth of these fibers. Some of the collaterals grow as far as the motor nerve cells themselves and complete two-neuron reflex arcs. This, it would seem, is the mechanism for reflexes of the spinal type.

The anatomical mechanism of the first reflexes consists of homolateral, unisegmental arcs. The first and principle development of these occurs in the brachial segments of the spinal cord. The reflex collaterals come off dorsal root fibers at the levels of entrance of these fibers where they can synapse with associational, rather than commissural, neurons. The associational neurons send their axons to motor nerve cells supplying the forelimb of the same side. In this way three-neuron reflex arcs are formed. Both two- and three-neuron arcs are developed coincident with appearance of the first stretch reflexes.

Longitudinal integration of one level in the spinal cord with another appears to be lacking at the time the first reflexes make their appearance. Longitudinal nerve fibers arising within the spinal cord course, for the most part, rostrad rather than caudad. Furthermore, long longitudinal tracts descending from the brain appear not to be established in embryos of the age in question. In regard to this, the observations from our laboratory are at variance with those of Angulo (1939), who has described an anatomical system of neurons in the rat fetus descending from the region of the midbrain and diencephalon, coursing in the ventral funiculus, and decussating ultimately in the floor plate of the spinal cord. This descending and crossing system of neurons is held to be the mechanism for integration within an expanding total pattern of behavior. Such a system would be very much like that which Coghill (1929*a*) found to exist in *Amblystoma*, where it constitutes the mechanism for mass type of movements in early development.[1]

Rhines and I (1941) have studied the development of the ventral and medial longitudinal fiber tract in various species of animals, including man. We found that descending neurons which constitute the true medial longitudinal fasciculus end in the medulla oblongata without decussating in early embryos and do not extend downward into the spinal cord. The ventral funiculus in the spinal cord and lower brain stem is made up of ascending fibers, predominantly, which overlap the descending fibers in the brain. This tract can, therefore, not be considered the mechanism for any total pattern of movement. More recent studies in the chick embryo by Rhines (1943) have shown that surgical removal of the rostral nucleus of the medial longitudinal fasciculus, preventing the downward growth of the tract which is claimed by Angulo to form the mechanism for the total pattern, has little effect upon the subsequent histologic picture of brain stem and spinal cord and that movements occur as in the unoperated controls. In the absence of any anatomical evidence for an integrating mechanism favoring a total pattern of mass movement and in the presence of anatomical proof of spinal reflex arcs which can explain the local, homolateral, spinal-type reflexes, it is time for a revaluation of the physiologic observations which led to the belief that mammals develop within a totally integrated system, as does *Amblystoma*.

Becker and I (Windle and Becker, 1940*b*) recently had occasion to re-examine the early stages in development of movements in the unanesthetized cat under physiologic conditions which were well controlled. The study confirmed our former views. The earliest somatic activities of which the cat embryo is capable make their appearance about 23 days after insemination. They are simple reflex responses to stimulation of the forelimbs or head. Additional simple responses appear during the following days of gestation, and these are elicitable independent of one another even after progressive integration of activities is accomplished.

By deliberately altering the quantity of oxygen or carbon dioxide breathed by the mother and by otherwise inducing asphyxiation in the fetuses, several pertinent facts were determined. We found that cat fetuses became more irritable to light tactile or pressure stimuli during the early stages of impaired respiratory conditions; the individuality of the responses was clearly defined at this time. It seemed that a partial anoxemia facilitated the occurrence of reflexes in embryos which normally were quiescent. With more prolonged anoxemia, individual movements became depressed. Some, especially those involving appendicular muscles, were lost entirely. Irritability decreased, stronger stimuli were required, and spontaneous movements resulted. Under conditions of asphyxia, stimulation of fetuses resulted in mass movements. The more profound the asphyxia, the more tonic and sustained were these mass movements. Complete oxygen lack, however, ultimately led to a breakdown of the mass response and to depression of all activities. Rhythmicity of certain movements, especially of the muscles to be employed later in respiration, appeared in fetuses about 30–31 days old under conditions of partial anoxemia. The more pronounced the anoxemia, the more muscles came into the respiratory-like rhythms of movement, until deep tonic dyspneic gasps alone occurred.

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[1] A. Cecil Taylor (*Anat. Rec.*, 87:379–413, 1943) has observed that in development of the frog "there seems to be no anatomical evidence for the assumption that localized behavior patterns of the limb are preceded by a generalized total pattern of true limb movement mediated through a special nervous mechanism."

These experiments demonstrated that conditions of respiratory metabolism are easily disturbed and that, when they are, individual or reflex-type movements are apt to be abolished before adequate stimuli can be brought into play to elicit them. Spontaneous mass discharges, misinterpreted by some as a totally integrated type of behavior, are often the only form of behavior remaining during partial anoxemia. When spontaneity is lacking, external stimulation induces similar mass discharges.

Very similar results have been obtained in human embryos (Fitzgerald and Windle, 1942). Movements were elicited at the operating table in unanesthetized, unnarcotized specimens of about 8 weeks' gestation. While the embryo was receiving oxygenated blood from the intact placenta, its neuromuscular mechanism was highly excitable. Movements which appeared to be individual reflexes were elicited by tapping lightly upon the amnionic sac. During progressive asphyxia these reactions ceased, but responses of the trunk musculature to stimulation of the nose and mouth region were obtained for several minutes after detaching the placenta from the uterus. Strong stimulation brought about mass discharges involving neck, trunk, arms, and legs. Here, too, in embryos of 22–26-mm. crown-rump length, we could readily induce movements by direct stimulation of the fetal muscles long after all reflexes had been abolished by asphyxia or when they were unelicitable because of general anesthesia.

It is interesting to note that one of the earliest reports of movements in human embryos of this age was that of Strassmann (1903), who observed activity of a specimen whose placental circulation was still intact and apparently functioning. He saw movements of the arms and legs. Only four human embryos smaller than 30-mm. crown-rump length have been observed by others to move at the time of operation, and asphyxiation was not avoided in any. Most older human fetuses have been asphyxiated during the period of experimentation, and it is the mass reaction during asphyxiation which the investigators have described and which led Coghill (1929*b*) to hypothesize a sequence of behavioral development for man like that in *Amblystoma*. Only when conditions are good, when anesthesia and anoxia are avoided, can one observe all of the reactions of which the fetus is capable and correctly interpret them.

Another study of the relation of anoxia to early reflex movements of cat embryos has been conducted in this laboratory

(Windle, Becker, Rhines, and Cowgill, 1942). Emphasis was placed upon movements of the head in response to stimulation of several points upon the face. Experiments were conducted without anesthesia on cats which had been decerebrated previously. At first the circulation of the fetal blood was maintained to the intact placenta while responses to stimulation were being observed. Later the umbilical cord was clamped, to induce asphyxia in the fetus.

The fetuses were relatively unresponsive when first delivered; but a partial anoxemia, setting in upon opening of the uterus, led to increased irritability in a few seconds or minutes. In the early stages of development, reflexes appeared after a short period of impaired oxygenation, although they had been absent a few seconds earlier. Reflexes were elicited at 23.5–25.0 days of gestation more readily by stimulating a large surface area and activating many receptors than by stimulating a point and activating only a few receptors.

During the course of development and before clamping the umbilical cord, reflexes resulting from stimulation of certain groups of receptors in the head region changed once or twice. For example, the preanoxic response to stimulating above the eye was a homolateral head movement at 26 days, changing to a contralateral movement at 36 days. In the same specimens during asphyxiation, the homolateral movement first appeared at 25 days. This changed to a contralateral movement at 27 days. The normal preanoxic reversals seem to come about in consequence of a fluctuating predominance of commissural or associational interneuron connections in the developing brain. The similar reversals occurring earlier under the influence of anoxia anticipated, as it were, the changes about to take place under better physiologic conditions. Anoxia enhanced the irritability of the neurons at first (a well-established physiologic principle) and ultimately abolished all reflex activity. It exerted some selective action in doing so, but the first reflexes to appear ontogenetically were not necessarily the last to disappear during anoxia.

Studies in the developing chick have contributed to our knowledge of genesis of somatic motor function. In early stages of incubation the chick brain stem is almost indistinguishable structurally from that of mammalian embryos of comparable size. In some, though not all, respects early somatic motility resembles that in the mammal. One cannot discuss the question of development of behavior without considering the very extensive chick studies of Kuo (1932, 1939*a*), which are frequently cited by other investigators.

Kuo's studies are impressive from the standpoint of the very large number of specimens which he used and the ingenuity of methods which he employed to study them. He coated the shell membrane at the blunt end of the egg with vaseline in order to render it transparent. It was implied that he could thus observe the developing chick throughout incubation. Unfortunately, the coating of vaseline impaired gaseous exchange, which normally takes place at the blunt end of the egg. Becker (1942) has demonstrated clearly that such treatment as Kuo used leads to abnormalities and to asphyxial death in most specimens. Kuo's results no more represent normal behavioral development than do those of investigators of mammalian species who used methods leading to asphyxiation.

I have considered the nature of early embryonic movements and their relation to the development of structures within the nervous system as well as their alterations by unnatural respiratory conditions and anesthesia. A number of other aspects of development of behavior might be discussed profitably. Some of these, as they pertain to later stages in development, will be found in articles by Carmichael (1933), Hooker (1938), and Barcroft and Barron (1937*b*, 1942). There is one other aspect to be considered at present because it bears a relation to the question of early development of motility.

If it is true that synaptic transmission in the central nervous system and neuromuscular transmission at end organs comes about through instantaneous production of acetylcholine, followed by sudden destruction by the enzyme choline esterase, one might expect significant changes in tissue concentration of these substances during periods of rapid functional development. This, indeed, seems to be the case.

Kuo (1939*b*) reported that acetylcholine appeared in chick embryos after 2–2½ days' incubation and that its concentration rose sharply through the fourth day. At about 4 days, chick embryos began to exhibit motility. From this time on to 12 days the concentration of acetylcholine ceased to rise and fluctuated within a fairly narrow range.

The enzyme choline esterase is present in fetal tissues in greater concentrations than in the adult. Nachmansohn (1940) found it highly concentrated in the region of nerve endings at an early age. In the spinal cord of sheep fetuses of 60–80 days gestation he demonstrated that the spinal cord contains more than the higher brain centers. Youngstrom's (1938) study of choline esterase in larval amphibians showed that the enzyme concentration started to rise during the early motile stages. A study of human fetuses by Youngstrom (1941) suggested a similar relationship. At between 56 and 120 days of gestation the enzyme concentration rose sharply in the lower centers (spinal cord, brain stem, and diencephalon), but only slightly in the higher centers (basal ganglia, cerebellum, and cerebrum), which are known to begin functioning relatively late. In the 56-day embryo (21 mm.) no part of the nervous system contained much enzyme. By 75 days (33 mm.) the concentration had risen strikingly. Since no intermediate stages were assayed, insufficient data were obtained to demonstrate as exact a relationship between choline esterase concentration and beginning nervous functioning in man as was found in the amphibians. Observations on movements in human embryos at about 8 weeks (Fitzgerald and Windle, 1942; Hooker, 1942) suggest that analysis of tissues from a graded series of specimens between 18- and 25-mm. crown-rump length might reveal a close correlation between the initiation of nervous function and the choline esterase concentration.

When one correlates these studies on choline esterase with others on direct observation of reflexes, especially in the later stages of development, it is found that a limited amount of generalization is possible. The mammalian fetus is essentially a spinal or bulbospinal animal in the early stages of development. As gestation proceeds, higher centers in the brain reach a functional state. Even at the time of birth, the cerebral cortex is immature, although great species variation is encountered. Recently Kennard and McCulloch (1942) explored the cerebral cortex of a 3-week-old monkey electrically and found that Area 4 was the only region from which responses were elicitable and that they were less discrete and of slower onset than in the adult. No responses were obtained from the leg region. Relatively high voltages were required for those from the face and hand area. This study offers one example of accomplishments that may be expected of investigations in the field of genesis of motor functions. Our knowledge of the full sequence of development in no species is complete. A great deal remains to be learned.

SUMMARY

Structural, mechanical, and chemical developmental factors are integrated to permit somatic motor functions to begin in mammalian embryos shortly before the time at which they take on external morphological characteristics of the particular species. The time at which movements begin spontaneously within the uterus is unknown, but it is probably days or even weeks after the first simple movement can be elicited by stimulation.

The first movement that can be obtained from an embryo depends upon a certain amount of maturation of its skeletal muscle fibers. Movements result from stimulating directly over growing muscle masses about 1 day before other movements which require the presence of functional nervous elements.

The first reflexes that can be obtained from any species of embryo are apparently of two types. Limb movements seem to occur in response to stretching the tissues or putting pressure upon them—i.e., muscle-stretch reflexes. These may require the presence of proprioceptive afferent neurons. The other type of reflex, represented by movements of the head which result from superficial stimulation even with light tactile stimuli in the face region, requires the presence of exteroceptive neurons. The limb reflex is a local homolateral movement, whereas the head reflex may be a contralateral movement. The difference between these two types of reflexes are clearly explained by structural differences in parts of the nervous system involved. A close correlation has been demonstrated between the time of appearance of the reflexes and the time of development and completion of reflex arcs in the nervous system. These reflex arcs are made up of two- and three-neuron chains.

Impairment of respiratory conditions in the embryo has a profound effect upon the early reflexes. In the first moments of a declining supply of oxygen to the embryo, elicitation of reflexes is enhanced or facilitated. As asphyxia sets in, however, reflexes change. A homolateral movement may become a contralateral movement, or vice versa. Rhythmicity of movement is another phenomenon occurring under duress. Some of the simple movements are abolished quickly by asphyxiation, and eventually all motility ceases. For some time before this occurs, it is relatively easy to bring about a mass discharge of nearly all motor units capable of functioning. This mass movement under duress of asphyxia is the type of movement which most early investigators saw when they examined fetuses which had been removed from the uterus. This is the movement which led to the belief that behavior in man has its genesis in a total pattern such as that in the larval amphibian *Amblystoma*.

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THE GROWTH OF THE CONTRACTILE VACUOLE IN AMOEBA PROTEUS

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It is a well-known fact that most contractile vacuoles arise by the fusion of accessory vacuoles, which previously arise *de novo* in the cytoplasm, the major exception being the canal-fed vacuole of the *Paramecium caudatum* group (for recent reviews see Kitching, 1938; Weatherby, 1941). Even in the case of the permanent and thick-walled contractile canal of the ciliate *Haptophrya*, the fluid is first segregated in the form of vacuoles in the doubly contoured walls (MacLennan, 1944). After the main vacuole has formed, two types of growth have been described—by the fusion of accessory vacuoles with the main vacuole or canal and by the intake of fluid directly through the membrane of the main vacuole. Both types have been demonstrated in live specimens of *Euplotes* (Taylor, 1923) and in six genera of the Ophryosoclecidae (MacLennan, 1933). Similar results have been reported in amoebae (Metcalf, 1910; Day, 1927; van Herwerden, 1927; de Saedeleer and Wolff, 1931; Wilber, 1942; etc.). Adolph (1926) and Mueller (1936), taking advantage of the normal spherical shape of the contractile vauole of *Amoeba proteus*, made quantitative studies of the growth of the vacuole and presented curves which show the growth of the main vacuole as a straight line. Adolph (1926, p. 363) interprets his curves as follows: "Figure 1 shows how, when plotted against time, the volumes indicated a uniform rate of increase and fell along a straight line." This conclusion that the increase is at a uniform rate has been widely accepted, and Kitching (1938) has utilized it in developing his secretion theory of diastole. However, the curve of uniform rate also implies that the accessory vacuoles, although present, contribute a negligible amount of fluid compared to the steady flow through the membrane, since the periodic fusion of the larger accessory vacuoles with the main vacuole would show up as sharply defined steps in the curve. Thus the curves, from the data of both of these authors, run counter to the morphological observations that large accessory vacuoles contribute a significant portion of the fluid discharged by the contractile vacuole.

The investigation reported below was designed to clarify the question raised by the work of Adolph and Mueller as to the relative importance of the contributions of the accessory vacuoles and of the membrane of the main vacuole. Both of these authors worked at room temperature, and their curves are based on relatively few observations in a single cycle. In the present investigation this difficulty was avoided by using a temperature of approximately 5° C., which greatly increases the length of the cycle and permits a sufficient number of accurate observations to determine the exact shape of the curve.

MATERIALS AND METHODS

The cultures of *A. proteus* used in this investigation are from a stock maintained in this laboratory for the last three years and originally derived from a culture furnished by the General Biological Supply House. They are maintained in a salt solution–rice culture according to the simple method of Chalkley (1930). The later and more complex methods of Chalkley and other authors have not been found to give any better results.

Elongate monopodal specimens were selected for all observations, both to give uniformity in the condition of individuals and to have the vacuoles most clearly visible. No selection for size was made. A small drop of the culture fluid containing the amoebae was placed on a slide inside a vaseline ring and the cover slip added. The preparation was always discarded unless a bubble of air was included. This method prevents any pressure from the cover slip from interfering with the amoeba, prevents changes in the osmotic pressure of the medium due to evaporation, and supplies enough oxygen for several hours' completely normal maintenance. However, none of the studies reported here were made on individuals which had been mounted more than 3 hours.

The ring mounts were sealed on the upper surface of a standard gas chamber for use on a microscope with hanging-drop preparations; but, instead of gas, water at the desired temperature was circulated. Thus the slide is in direct contact with the cooling agent, light is transmitted through the cooling medium, and the specimen is viewed under good conditions with an oil-immersion lens. Water was sent into the chamber at a temperature of 3° C. and emerged at 8° C., so that the temperature of the slide itself was close to 5° C.

All measurements were made with a camera lucida by combining the images of the vacuole and of a scale marked in microns placed in the usual drawing position. This scale was made by projecting the image of an engraved slide, and the spaces of 10 μ were then subdivided to 1 μ. The magnification used was 1,600. By using separate lamps for the microscope and for the camera lucida, the images of both the vacuole and the scale were sharp. For timing the 15-second intervals, a watch with a prominent second hand was placed in the field of the camera lucida so that its image appeared in the microscope field close to, but not on, the image of the vacuole. With this arrangement, measurements can be made easily and accurately within 5 seconds, the remaining 10 seconds of the intervals of 15 seconds used in this investigation were available for recording measurements, adjusting the apparatus, etc.

The vacuole of *A. proteus* is spherical, and the volumes were calculated from the measured diameters on this basis. The boundary between the fluid of the vacuole and the surrounding collection of granules and special cytoplasm was used, so that the volumes reported include only the volume of fluid in the main vacuole and do not include any of the cytoplasm or any adjacent vacuoles. Diameters were recorded to the nearest $\frac{1}{2}$ μ; and, in the rare cases where the vacuole was not quite spherical, two axes at right angles were measured and the average taken. The microscope was carefully focused immediately before each measurement was made, to be sure that a true diameter and not a chord was being measured.

In addition to measurements of the diameter of the main vacuole, the time of the end of the systole of the previous cycle (zero time on the graphs) and the time of the appearance of the first vacuole (marked by an arrow on the graphs) were recorded.

OBSERVATIONS

After the amoebae were mounted in the manner described above, they were kept at room temperature until normal, active locomotion was well under way. The cold water was then circulated through the chamber and observations started. The first response of the amoeba may be termed a shock reaction—streaming ceases for several minutes, then slowly starts, first in one direction and then in another. After 15 minutes, streaming usually becomes continuous, and locomotion in a particular direction is maintained for several minutes at a time. All records reported here were made after normal locomotion had been re-established and include only those vacuolar cycles which were completed before any marked change in the direction or speed of locomotion occurred. The movements are slower, of course, than at room temperature, and the amoeba is somewhat shorter and broader, but in all other respects it appears and behaves the same as at room temperature. The temperature selected slows the amoeba but does not disrupt its normal behavior, once the initial shock period has passed.

The diameter of the main contractile vacuole of *A. proteus* at 5° C. remains constant except for fluctuations of less than 0.5 μ for periods of 15 seconds up to several minutes; then a sudden increase takes place in a period of 3–10 seconds, followed by another quiescent period; and the process is repeated until the vacuole is fully grown and systole takes place. When the volumes calculated from these successive measurements of the diameter are plotted against time, the growth curve resulting (Figs. 1–3) is in all cases made up of a series of sharply defined steps. Since both the size of the accessory vacuole and the frequency of formation tend to be uniform, the general trend of the curve is toward a constant absolute growth as reported by Adolph (1926) and Mueller (1936); but this growth is accomplished by periodic increments, not by the continuous addition of fluid to the main vacuole.

The regularity of the general trend of the curve may be interrupted in at least three ways. Adolph (1926) has described many cases of a change of rate during the vacuolar cycle, which is shown in the stepped curve by an increase in the size of the increments and a decrease in the time between steps. Partial systole (Fig. 2) may occur, after which the small vacuole which remains grows again in the usual manner. Finally, some curves (Fig. 3) show one or two steps which are several times greater than the average of the other steps, the general rate before and after this large step being the same.

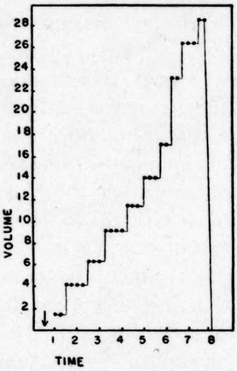

FIG. 1.—A common type of growth curve of the contractile vacuole of *Amoeba proteus*. Volume is expressed in thousands of cubic microns; time in minutes. Temperature is 5° C. The arrow indicates the time at which vacuoles first become visible.

In one series of observations, most of the time was used in studying the structure of the vacuolar complex, and only enough measurements were made to prove the start of each new step. It was found that the new step was the result of the fusion of one or more accessory vacuoles with the main vacuole. The accessory vacuoles are easily observed in the early stage of the cycle while the main vacuole is still small; but the later ones are often obscured by the main vacuole and are not found unless a careful search for them is made or unless they happen to lie lateral to the vacuole instead of below or above it. Day (1927) was most interested in the early stages of the vacuole, and this probably accounts for his lack of emphasis on the accessory vacuoles in the later stages of diastole. These accessory vacuoles are readily seen, even when taking measurements, if they happen to lie in the plane being used for measurements; and in the case of the large step shown in Figure 3 three large vacuoles were seen to fuse with the main vacuole. Further observations on other individuals showed that in most of the steps only one accessory vacuole fuses with the main vacuole; but several more cases of multiple accessory vacuoles were found. It should be noted that these statements apply to the part of the vacuolar cycle in which one main vacuole is established, since in the early stage many accessory vacuoles are typical. The multiple accessory vacuoles in the later stage may be due at times to a sudden increase in the rate of segregation of

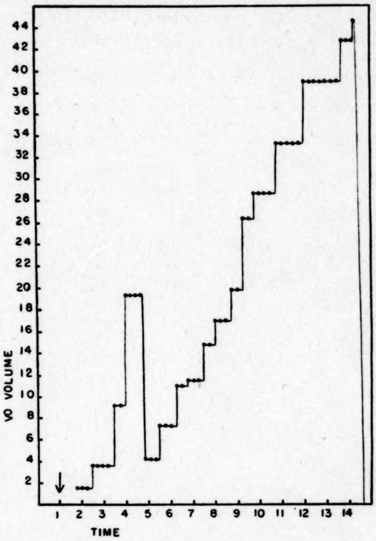

FIG. 2.—The growth curve of a vacuole which underwent partial systole in the early phases. For explanation of symbols see Fig. 1.

fluid by the vacuolar complex; but in several cases (as in Fig. 3) the usual serial formation of accessory vacuoles occurred. However, some of the early ones did not fuse with the main vacuole as soon as they were formed but fused later along with the newer vacuoles. In these cases, then, the break in the curve means a change in the rate of fusion of the accessory vacuoles with the main vacuole, rather than a change in the rate of segregation of fluid.

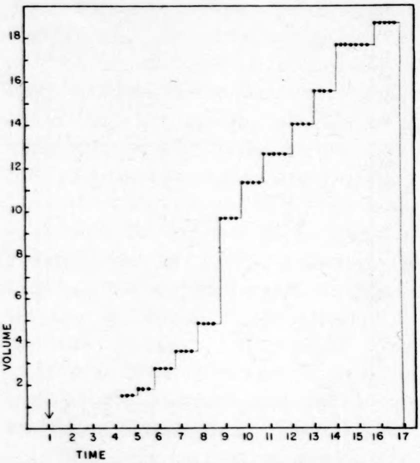

FIG. 3.—The growth curve resulting from the delayed fusion of some accessory vacuoles with the predominant vacuole. For explanation of symbols see Fig. 1.

Before the fusion of most of the accessory vacuoles to form a single predominant vacuole (referred to in this paper as the "main vacuole"), no accurate measurements of the fluid segregated can be made. Extrapolation of the curve to cover this early portion of the cycle can be accomplished by adding steps of average size, since, as shown above, the steps tend toward a regular progression. Inspection of Figures 1 and 2 shows that in each case one average step would be sufficient to bring the curve to zero volume, and this would also intersect the abscissa close to the point which represents the first appearance of visible vacuoles (marked by the arrow). In Figure 3 the simple addition of one or perhaps two steps would bring the volume to zero but at a point representing a lapse of not less than 2 minutes after the first appearance of the accessory vacuoles. However, delayed fusion of vacuoles was described for this particular cycle as well as for other cylces, so that it is most probable that the first segment of the curve represents the volume not of the whole vacuolar complex but of only a part. If extrapolation is started from the main segment of the curve, the apparent discrepancy between this cycle and that of the first two cycles disappears, since the extrapolated series of steps extends to the point at which the accessory vacuoles first become apparent. Since the extrapolated curve and the time of appearance of the accessory vacuoles coincide, the assumption that the rate of segregation of fluid is the same before the formation of the main vacuole as after the formation of this vacuole fits the observed facts.

The lag period between the time of the end of systole of the previous cycle and the first appearance of accessory vacuoles was variable and ranged from 15 seconds to 5 minutes in this series of experiments, the commonest time being 1 minute. The variability seems to be due to individual differences between amoebae, since the lag time tends to be quite constant in successive cycles in the same individual. A final lag period or resting period, in which no change in the volume of the vacuole occurs over a relatively long time, is found in many ciliates (Taylor, 1923; MacLennan, 1933), but no such lag period occurs in *Amoeba*. This is correlated with the fact that in these ciliates accessory vacuoles for the next cycle form even while the vacuole of the present cycle is still there; but in *A. proteus* all accessory vacuoles fuse with the main vacuole right up to the time of systole.

This morphological and qualitative summary of the growth curve of the contractile vacuole shows that the characteristics of the whole curve are a result of the summation of the characteristics of the individual steps. In addition to the individual observations reported, a further analysis (Table 1) was made of 87 steps in 10 vacuolar cycles chosen because the amoebae in which they occurred were all taken from the same culture within the same week and thus were as close to a homogeneous group as can be obtained with amoebae. To bring out the relationship between the size of the vacuole and the characteristics of the steps, each step is tabulated according to the diameter of the vacuole in the quiescent period before the phase of increase, arranged in groups covering a range of 5 μ.

TABLE 1

SUMMARY OF THE STEPS IN TEN VACUOLAR CYCLES IN SIX INDIVIDUAL AMOEBAE TAKEN FROM THE SAME CULTURE

		DIAMETER OF THE VACUOLE AT THE START OF EACH STEP (μ)				
		15.5–20.0	20.5–25.0	25.5–30.0	30.5–359.	35.5–40.0
Increase in diameter (μ)	mean	2.8	2.5	2.2	1.8	1.2
	range	0.5–7.0	1.0–5.5	1.0–6.5	0.5–6.0	0.5–2.0
Increase in volume (μ^3)	mean	2,000	2,200	3,000	2,600	2,900
	range	700–3,300	1,000–4,900	1,000–3,400	1,000–6,600	1,000–5,600
Duration of step (seconds)	mean	40	45	52	45	57
	range	15–60	15–75	15–120	15–105	30–120
Number of steps		14	14	18	21	20
Mean increase per step per mean duration		50	44	58	58	58

The increases in volume were calculated for each step and were not approximated from the data on the mean increase in diameter.

The mean increase in diameter is greatest in the small vacuoles and drops steadily as the size of the vacuoles increases. However, the range of measurements is nearly the same, except in the group of vacuoles of the largest diameter. The mean increase in volume for all the steps is 2,600 μ^3, with the smaller vacuoles tending to show a smaller increase than the larger ones. The mean duration of time between increases in volume is 49 seconds; and in this characteristic also there is a tendency for the lower values to be associated with the smaller vacuoles. When the mean increase in volume is divided by the mean time per step to give the rate of increase in the accessory vacuoles which formed those steps, some of the fluctuations were lost and the rest reduced in amount so that, while there is a tendency for vacuoles below 25 μ in original diameter to be in a separate group from those over 25 μ in original diameter, the rate of increase shows that the separation between the two groups is smaller than when only the increase in volume is considered. The mean rate of increase for all the steps was 53 μ^3/sec. at 5° C., as compared to a mean value for the vacuole as a whole (including the initial lag period) of 88 μ^3/sec. at 18°–27° C., as reported by Adolph (1926). When allowance is made for the difference in temperature, it is seen that these results are very close and that the two groups of amoebae used are comparable.

The time which elapses between the start of the fusion of an accessory vacuole with the main vacuole and the resumption by the main vacuole of its usual spherical shape is usually less than 5 seconds, although in a few cases this took as long as 10 seconds. Since the mean time between the sudden increases in size is 49 seconds, the time occupied by the fusion of the vacuoles is usually not more than 10 per cent of the total time from the start of one step to the start of another, so that this latter interval can be considered as substantially the same as the period between the end of one fusion process and the start of the next one, which has been called in this paper the "quiescent period."

In this quiescent period no change in the diameter of the vacuole could be detected when measured to the nearest $\frac{1}{2}$ μ. As shown by Table 1, changes of only $\frac{1}{2}$ or 1 μ occur in all size groups and become more common in the larger vacuoles. The experimental errors in these cases of small changes are relatively high and in the larger vacuoles are magnified relatively more in calculating the volumes. From the standpoint of the group as a whole, the evidence for the presence of steps in the growth of the vacuole is strongest in the smaller vacuoles and weakest in the largest ones. However, in some vacuoles, changes in diameter at each step are large throughout the cycle, and in one such vacuole the measurements for the successive steps were 14, 19, 26, and 32.5 μ, followed by systole. These large changes, which are always present in some vacuoles, present in at least some of the steps in many vacuoles, and rarely completely lacking, are far larger than any possible experimental error.

The only variations which could be detected in vacuoles which were not obviously deformed by the pressure of the plasmasol stream or by contact with large cytoplasmic structures were less than 0.5 μ. In one amoeba which was well spread out, and with the cytoplasmic inclusions fewer than usual, so that visibility was unusually good, the size of the vacuole was estimated to tenths of a micron during a period when the vacuole was held motionless in the plasmagel. In this case the measurements for one unusually long step were recorded as follows: 36.9, 37.0, 37.0, 37.0, 37.0, 37.2, 36.9, 37.2, 37.0, and 37.2 μ. The largest measurement in the step previous to the one quoted was 35.5 μ, and the smallest measurement in the succeeding step was 38.8 μ. Even under the best conditions, estimating tenths of a micron is pressing the method beyond its limits of accuracy; but it is significant that no variations are detected in the vacuole between the sudden increases in size unless the units of measurement used are smaller than justified by the accuracy of the methods used.

There must be considered the possibility that the steps are induced in some way by the low temperature or by the sudden change in temperature and are not present when kept constantly at the temperatures (18°–27° C.) used by Adolph and by Mueller. This possibility was investigated by making observations in a room isolated from the rest of the building and kept at 23° ± 1° C. night and day during the course of these experiments. Some of the amoebae used were taken directly from the stock cultures, and others were the same individuals and in the same amounts used for the observations at low temperature. Steps were found in both of these series, some of about 15 seconds' duration, many of 30 seconds; and a few of longer duration. No difference was found between curves from these observations and the ones from the observations at low temperature, except that the steps in the former are shorter and therefore less convenient to demonstrate. This is confirmed by Adolph's figures, in many of which the points do not progress in a constant manner but tend to be arranged in flat steps.

DISCUSSION

The growth curve of the contractile vacuole of *A. proteus* is made up of a series of steps which are the result of the periodic fusion of the accessory vacuoles with the main vacuole. In the quiescent periods between the rapid, large increases in volume, no change could be found, the measurements of diameter being made to the nearest ½ μ. The characteristics of the quiescent period are important; for, if there is actually no change in volume at these times, it means that all the fluid is segregated by the accessory vacuoles and that the membrane of the main vacuole has no function in the segregation of fluid.

The magnitude of the increase in diameter at each step is, on the average, greater in the smaller vacuoles than in the larger ones; but the actual increase in the volume at each step and the duration of the step increases only a small amount as the size of the vacuole increases. Thus both the steps involving a large increase in diameter and those involving a small increase in diameter are a result of the same processes and of processes of the same order of magnitude. Therefore, conclusions reached from an examination of the steps which involve a large increase in diameter in which the relative accuracy is greatest can be applied to all the steps.

In the case of a step which involved a sudden increase to 26 μ after a quiescent period of 45 seconds in which the diameter was 19 μ, if 20 per cent of the fluid entered by diffusion across the main vacuolar membrane rather than by way of the accessory vacuoles, there would have been a change from 19 to almost 21 μ during the quiescent period. Since such a movement across the membrane would be continuous, there would be a continuous increase in diameter, which, however, because the measurements were made in units of 0.5 μ, would show up as a series of small steps followed by the large step caused by an accessory vacuole, and the whole curve of vacuolar growth would be composed of several such series. This was not the case in the particular vacuole used as an example, and actually no case of this sort was found. If the error in measuring this particular quiescent period was 0.5 μ, only 5 per cent of the fluid could have entered the vacuole by the action of the membrane. However, even this small figure is unlikely, since, if it did occur, extremely long quiescent periods would be lacking, because an even smaller rate over a long period would raise the diameter measurably. In the group of steps tabulated, resting periods of 120 seconds were found, and in other vacuoles not included in that summary a few periods of 240 seconds were observed. In all of these, as well as in the shorter periods, no increase in size could be measured. Thus, if any appreciable amount of fluid entered through the membrane rather than from the accessory vacuoles, it would produce a different pattern in the curve of growth than the relatively regular series of steps actually observed.

The main vacuole, therefore, is simply a reservoir for fluid previously segregated in the accessory vacuoles and is a part of the mechanism for the expulsion of this fluid to the outside. The lag period between the end of systole and the appearance of accessory vacuoles suggests that the vacuolar fluid is first accumulated in restricted cytoplasmic regions and is then separated from the surrounding cytoplasm by the formation of a membrane. The membrane of the accessory vacuole, according to this view, is a result of the localized accumulation of fluid, not its cause. The morphological aspects of such a situation have been described in *Amoeba* (Day, 1927; van Herwerden, 1927; de Saedeleer and Wolff, 1931), and in various ciliates (Taylor, 1923; MacLennan, 1933).

Kitching (1938), in a series of experiments on salt-water ciliates, developed a secretion theory of diastole and states that "water is secreted into the contractile vacuole by the vacuolar membrane itself, but solutes are in general not able to enter the vacuole from the cytoplasm." He also feels that his evidence would support the idea that the vacuolar fluid is very close to pure water, at least hypotonic to the cytoplasm. Krogh (1939) agrees in general with Kitching's theory but finds it difficult to construct a membrane which would have the properties that Kitching's theory demands. However, the results presented in this paper show that Kitching's secretion theory must be modified in so far as *A. proteus* is concerned by substituting for the membrane itself the cytoplasm, presumably a specialized portion of cytoplasm adjacent to the vacuole. There is such a specialized cytoplasmic region, which consists of granules (Metcalf, 1910) and a layer of viscous protoplasm (Mast and Doyle, 1935; Mast, 1938); and it may be that this material is the active agent in the segregation of fluid. This region has many of the cytological characteristics of secretion and is similar to the specialized vacuolar regions of ciliates, whose structural adaptations for secretion have been stressed previously (for reviews of this subject see Weatherby, 1941; MacLennan, 1941). Interesting experiments involving the removal of various amounts of these granules (Mast and Doyle, 1935) show that the activity of the vacuole decreases as the amount of granules decreases; but the interpretation that the granules are necessary for the functioning of the vacuole has been questioned recently (Mast, 1938) on the ground that in normal specimens the number of granules cannot be correlated with the activity of the vacuole. Further experiments are needed to clarify this point.

Although the evidence is clear that in *A. proteus* no important quantity of fluid is segregated by the action of the membrane of the reservoir vacuole, it is not intended to make this a generalization applicable to all vacuoles. I have observed in the ciliate *Eudiplodinium* a large increase in the size of the contractile vacuole during late diastole, even though no accessory vacuoles form after early diastole (MacLennan, 1933, Fig. *D*, 4, 5, 6). In the ciliate *Haptophrya* (Bush, 1934; MacLennan, 1944), the vacuolar wall is thick, permanent, and doubly contoured. Obviously, all the fluid diffuses through the outer boundary of the wall and is then segregated in vacuoles, the contents of which empty into the lumen of the canal by fusion with it. Thus in the same excretory system, one surface of the membrane is sufficiently permeable to transfer all the water, while in the other surface the transfer of water into the lumen is by visible ruptures. These different examples may involve only superficial differences rather than differences in the basic physiology; but they do serve as a reminder to be cautious in making sweeping generalizations about the contractile vacuole from only a single case.

SUMMARY

1. The details of the growth curve of the contractile vacuole of *A. proteus* were studied at 5° C., a temperature which slows the vacuole but does not otherwise affect the cycle.

2. This growth curve is made up of a series of sharply defined steps and is never a smooth curve.

3. The accessory vacuoles are the important sources of fluid in the main vacuole, and at most only a negligible portion of the fluid is segregated by the membrane of the main vacuole.

4. The presence of a lag period after the systole of the previous pulsatory cycle and the formation of new accessory vacuoles is interpreted as indicating that an important phase of the segregation of fluid is accomplished by the cytoplasm before the formation of any membranes.

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PHOTODYNAMIC ACTION IN THE FLAGELLATE PERANEMA TRICHOPHORUM WITH SPECIAL·REFERENCE TO MOTOR RESPONSE TO LIGHT[1]

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RAAB (1900) discovered that when paramecia are put into a solution of acridine they die much more quickly if the solution is exposed to light than if it is kept in darkness. Von Tappeiner and Jodlbauer (1904), in whose laboratory the work was done, designated this interaction between dyes and light "photodynamische Erscheinung." Raab's discovery led to extensive further investigations in this field, with various dyes and numerous organisms and cells, concerning this phenomenon.

These investigations have recently been reviewed by Blum (1941). Nearly all of them, like those made by Raab, concern the injurious effect of light on organisms and cells in solutions containing dyes. In a few of them it was, however, observed that motor response to light can be induced by treatment with dyes. This has been observed in muscles and nerves, in unicellular organisms, and in roots of seedlings.

Adler (1919), Lippay (1929, 1930), Spealman and Blum (1932), and Lillie *et al.* (1935) found that muscles which ordinarily do not respond to light, contract if they are exposed in solutions of certain dyes. Metzner (1920, 1921) discovered that bacteria, paramecia, and a few other unicellular forms which are neutral to light in ordinary culture fluid respond very definitely with the "motor reflex" if erythrosin or eosin is added (orientation was not observed). Metzner (1923) observed that if roots of oats and radish seedlings are laterally illuminated in culture fluid containing erythrosin the tips turn toward the light; and Blum and Scott (1933) observed the same in wheat seedlings.

Metzner's observations are the only ones that have been made on the effect of dyes on motor responses of entire organisms to light, and these observations are merely qualitative. It is consequently obvious that observations on the quantitative relations between various dyes and motor responses of organisms to light are highly desirable. The following report concerns such observations made on *Peranema trichophorum*.

MATERIAL

Peranema trichophorum is a colorless flagellate without an eyespot. It is a favorable subject for the experiment because it can be readily raised in the laboratory in darkness, moves slowly, and responds very definitely to a sudden increase in illumination. Mast and Hawk (1936) and Shettles (1937, 1938) investigated various phases of this response.

The peranemae used were obtained as follows: Specimens from a mixed protozoan culture were transferred to finger bowls, each of which contained 100 ml. of modified Chalkley solution (hereafter referred to as "culture fluid") and two grains of rice. The solution consisted of NaCl, 80 mg.; $CaCl_2$, 4 mg.; $NaHCO_3$, 4 mg.; KCl, 4 mg.; $Ca(H_2PO_4)_2$, 2 mg.; Na_2HPO_4—M/15, 2 ml; KH_2PO_4—M/15, 3 ml.; distilled water, 1 liter.

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[1] I wish to express my appreciation to Professor S. O. Mast for suggesting the problem and for generous assistance concerning the experimental work and the preparation of the manuscript.

From these stock cultures smaller working cultures were prepared in essentially the same way. Stender dishes of 30-ml. capacity were half-filled with the culture fluid, then one grain of rice and a number of peranemae were added, after which the cultures were put into light-tight boxes and left for two weeks, i.e., until the peranemae were abundant. The purpose of this procedure was to produce a uniform state of dark adaptation. It was not deemed necessary to produce maximum sensitivity to light, which, according to Mast and Hawk (1936), occurs after only one hour of dark adaptation.

METHODS

The experiments were performed in a darkroom equipped with a ventilated dark chamber, which contained a 500-watt monoplane-filament projection lamp in a 110-volt circuit. The lamp was mounted back of an opening 3 cm. square in the wall of the dark chamber, and a sliding shutter carrying a red-glass filter was mounted in front of it. A condensing lens and a water-cell 4 cm. thick were mounted in front of the shutter. A microscope equipped with a mechanical stage was placed in the beam of light, in front of the wall of the dark chamber, and adjusted so that the beam was reflected upward from the plane surface of the substage mirror. The intensity of the light was 20.35 meter candles at the level of the stage. The light was measured with a Weston photometer.

The temperature of the room was maintained at 22° ± 0.5° C. The voltage in the circuit supplying the lamp was maintained at 105 volts during each experiment, by means of a variable resistance.

ABSORPTION OF LIGHT BY THE DYES USED[2]

The absorption of light in a dye solution is dependent, to some extent, on the character of the solvent. All the dyes used were therefore dissolved in culture fluid at a standard concentration of 2×10^{-5} M. The absorption in various regions of the spectrum was measured with a Coleman spectrophotometer. The dyes used and the results obtained are given in Table 1.

This table shows that for all the dyes used the region of absorption in the spectrum extends over a wide range but that the percentage of absorption increases to a maximum, as the wave-length increases and then decreases, and that in two of the dyes there is a secondary maximum. It shows that the region of maximum absorption varies from about 4200 Å for auramine O to about 6300 Å for methylene blue and that for concentrations of 2×10^{-5} M the absorption in this region varies from about 65.3 per cent for neutral red to 100 per cent for rose bengal and brilliant green.

RELATION BETWEEN REACTION TO LIGHT AND THE CONCENTRATION OF THE DYE

Rose bengal.—A Stender dish containing a vigorous culture of peranemae was selected and the culture fluid poured out. The dish was rinsed with a small quantity of rose bengal, 1×10^{-5} M in culture fluid, to remove the remaining fluid and then half-filled with the dye solution.

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[2] All the dyes used were obtained through the courtesy of the National Aniline and Chemical Company. The same glass cell was used to contain each dye solution, and it was always oriented in precisely the same way in relation to the source of light; hence the thickness of the layer of dye solution being examined was invariable.

Several peranemae (10–12) were then, with a capillary pipette, picked up from the bottom, to which they cling, and deposited on a clean glass slide and covered with a cover glass supported by a vaseline ring.[3] (If the vaseline rings are put on the slides in advance, a large number can be prepared in a few minutes.) Twenty-four additional slides were then prepared in the same way. These slides were put into a light-tight box and left for 1 hour; then each slide was put on the stage of the microscope, and an actively crawling peranema was brought into focus in red light, after which, by dropping the shutter in front of the opening in the dark chamber, it was suddenly exposed to white light (20.35 m.c.) and its reaction-time measured with a stop watch.[4] This was repeated with each of 49 other dark-adapted specimens, with 50 in each of a number of different concentrations of rose bengal, and with 200 in culture fluid without added dye.

TABLE 1

THE RELATION BETWEEN THE WAVE LENGTH AND THE PERCENTAGE OF ABSORPTION OF LIGHT BY VARIOUS DYES, 2×10^{-5} M, IN CULTURE FLUID

Dyes 2×10^{-5} M	Percentage of Absorption at Various Wave Lengths (In Angstrom Units)											
	3800	4100	4400	4700	5000	5300	5600	5900	6200	6500	6800	7100
Rose bengal	16.5	20.0	27.8	57.0	92.0	100	94.3	7.0	0.8	0.5	......	......
Neutral red	......	43.0	57.5	63.3	64.3	65.3	51.5	27.0	15.2	11.0	10.0	9.0
Eosin	11.0	14.8	25.0	70.0	96.3	86.3	4.5	......	......	......	......	......
Methylene blue	6.0	3.5	6.0	13.0	17.5	25.0	51.0	83.0	94.0	98.5	83.0	13.0
Nile blue sulphate	15.0	12.0	9.0	16.0	41.5	75.0	92.5	95.5	90.8	37.5	8.5	3.5
Auramine O	49.0	73.6	79.5	31.0	2.0	1.0	......	......	......	......	......	......
Orange G	38.0	44.8	61.5	81.0	76.5	15.0	......	......	......	......	......	......
Brilliant green	60.5	55.5	6.0	14.5	37.5	71.3	93.2	99.4	100	......	......	......

The results obtained are presented in Table 2. In this and the following experiments, concentrations of dye greater than those recorded in the table are lethal.

Table 2 shows that the average reaction-time of the 200 peranemae in culture fluid without dye was 12.1 seconds and that it was nearly the same for the solutions of rose bengal, 5×10^{-8} M and lower, but that as the concentration of the dye increased from 5×10^{-8} M, the average reaction-time decreased to a minimum of 0.92 seconds, at 2×10^{-5} M, then increased slightly,[5] i.e., to 0.96 seconds at 5×10^{-5} M, and then became lethal at 1×10^{-4} M.

This indicates that rose bengal has no effect on the reactions of *Peranema* to light if the concentration is lower than 5×10^{-8} M but that it has a great effect in optimum concentrations and that the effect is closely correlated with the concentrations.

Table 2 shows also that, as the concentration of the dye increased, the

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[3] All operations except the sudden exposure to white light were carried out in weak red light which does not stimulate *Peranema* (Shettles, 1937). The intensity was very low in order to avoid any harmful effects due to pre-irradiation of those dyes which absorb the longer wave lengths.

[4] The reaction-time varies inversely with the intensity of the light which induces it (Shettles, 1937). The relatively low intensity of the stimulating light was selected so as to obtain a reaction-time which could be fairly accurately measured with a stop watch.

[5] The standard error of the difference between 0.92 and 0.96 seconds is ±0.01965.

TABLE 2

THE RELATION BETWEEN THE CONCENTRATION OF DYES AND THE AVERAGE REACTION-TIME OF *Peranema trichophorum* SUDDENLY SUBJECTED TO 20.35 M.C.

Each reaction-time is the average for 50 peranemae (one measurement for each) except the last one in the first column, which is the average for 200. L = Lethal; Reac.-time = Reaction-time; Std. dev. = Standard deviation. Orange G, 2×10^{-4} M, is slightly toxic, and more concentrated solutions are lethal. Solutions of brilliant green less concentrated than 1×10^{-3} M have no observable effect on *Peranema*.

Molecular Concentration of Dyes	Rose Bengal		Neutral Red		Eosin		Methylene Blue		Nile Blue Sulphate		Auramine O		Orange G		Brilliant Green	
	Reac.-Time (In Seconds)	Std. Dev.	Reac.-Time (In Seconds)	Std. Dev.	Reac.-Time (In Seconds)	Std. Dev.	Reac.-Time (In Seconds)	Std. Dev.	Reac.-Time (In Seconds)	Std. Dev.	Reac.-Time (In Seconds)	Std. Dev.	Reac.-Time (In Seconds)	Std. Dev.	Reac.-Time (In Seconds)	Std. Dev.
2×10^{-4}											L		10.55	7.32		
1×10^{-4}	L		L		L		L				5.59	4.78	11.98	11.32		
5×10^{-5}	0.96	0.11	1.04	0.22	1.64	0.37	1.50	0.77	L		5.48	4.47	10.14	11.48		
2×10^{-5}	0.92	0.09	1.10	0.24	1.40	0.22	1.92	0.75	4.14	5.13						
1×10^{-5}	1.10	0.01	1.47	1.54	1.40	0.27	2.08	0.98	6.14	5.74	6.41	7.18	12.53	12.79		
5×10^{-6}									9.10	9.78					L	
2×10^{-6}	1.20	0.02	2.02	1.16	2.11	0.92	5.40	5.08	11.20	14.81					16.24	14.83
1×10^{-6}	1.70	0.40	4.22	6.09	2.48	1.36	7.80	10.78			9.65	8.66	11.50	13.08	14.93	14.93
5×10^{-7}	1.89	0.53	10.03	12.74	8.06	7.97	8.64	10.91								
2×10^{-7}	2.84	2.43	7.67	9.70	14.80	15.88									13.19	12.83
1×10^{-7}	8.17	9.66	10.54	12.14	12.02	13.56	11.80	16.50	12.46	14.39	13.10	12.47			12.43	13.61
5×10^{-8}	13.17	12.43	12.53	14.71	12.24	11.13										
2×10^{-8}	13.41	14.27	12.20	9.50	13.02	11.70										
1×10^{-8}	13.24	13.43	13.57	14.96	13.70	12.60	10.90	14.59	12.11	13.41	13.92	12.78			12.61	13.19
0	12.10	13.60														

standard deviation decreased from about 14 at 1×10^{-8} M to about 0.1 at 5×10^{-5} M. This shows that, if the concentration of dye is sufficient to reduce the reaction-time significantly, it produces a highly uniform behavior on the part of the peranemae.

Methods like those described above were used to ascertain the effects of seven other dyes. The results obtained are given in Table 2 and are discussed below:

Neutral red.—The reaction-time increased from 1.04 seconds at 5×10^{-5} M to 13.57 seconds at 1×10^{-8} M, and the standard deviation from 0.22 to 14.96.

Eosin.—The reaction-time decreased from 1.64 seconds at 5×10^{-5} M, to 1.4 at 2×10^{-5} M; then increased—slowly at first, then rapidly—to 14.8 seconds at 2×10^{-7} M, after which it remained fairly constant. The standard deviation decreased from 0.37 at 5×10^{-5} M to 0.22 at 2×10^{-5} M, then increased to 15.88 at 2×10^{-7} M, and then declined slightly.

Methylene blue.—The reaction-time increased from 1.5 seconds at 5×10^{-5} M to 11.8 seconds at 1×10^{-7} M, then decreased very slightly to 10.9 seconds at 1×10^{-8} M. The standard deviation increased from 0.77 at 5×10^{-5} M to 16.5 at 1×10^{-7} M, then dropped to 14.59 at 1×10^{-8} M.

Nile blue sulphate.—The reaction-time increased from 4.14 seconds at 2×10^{-5} M to 12.11 seconds at 1×10^{-8} M. The standard deviation increased from 5.13 at 2×10^{-5} M to 14.81 at 2×10^{-6} M, then decreased slightly.

Auramine O.—The reaction-time increased from 5.59 seconds at 1×10^{-4} M to 13.92 seconds at 1×10^{-8} M, and the standard deviation from 4.78 to 12.78.

Orange G.—The reaction-times measured for various concentrations of orange G vary so little from those of peranemae in culture fluid alone that it seems probable that this dye has no photodynamic action, at least under the conditions of this experiment.

Brilliant green.—The reaction-time decreased from 16.24 seconds at 2×10^{-6} M to 12.61 seconds at 1×10^{-8} M; but the standard deviation was approximately the same at all concentrations.

DISCUSSION

It has been suggested by earlier workers that photodynamic action is correlated with fluorescence, molecular structure, and the wave length of the light which is absorbed by the dyes. These views and others are considered below.

Fluorescence.—Many of the photodynamically active dyes are noticeably fluorescent. There have consequently been attempts to show that photodynamic action is caused by fluorescence, but very little evidence has been found to support this view. Raab (1900), for example, found that if parameciae in a weak solution of eosin are exposed to light they are killed but that fluorescent radiation from an eosin solution with which the animals are not in contact produces no ill effects.

The dyes used in the present experiments vary greatly in degree of fluorescence. According to Metzner (1927), the comparative degree of fluorescence is as follows: eosin, strong; rose bengal and neutral red, weak; Nile blue sulphate and methylene blue, very weak; auramine O, none. Brilliant green and orange G, not listed by Metzner, are not visibly fluorescent under ordinary conditions.

Table 3 shows that there is a general correspondence between the degree of fluorescence of the dye used and the effect on the sensitivity of *Peranema* to light but that there are exceptions to this correspondence; for auramine, which is not fluorescent, is fairly effective. The facts in hand, therefore, indicate that fluorescence, per se, is probably not the cause of photodynamic action.

Molecular structure.—Photodynamic reactions have been produced by the use of a large number of dyes (Metzner, 1927; Tennent, 1935–38, 1942; Blum, 1941). A study of the structural characteristics of these dyes (Conn, 1940) makes it clear that no specific molecular structure is required to produce the phenomenon.

TABLE 3

THE RELATION BETWEEN VARIOUS PROPERTIES OF DYES AND THEIR EFFECT ON THE RESPONSE OF *Peranema* TO LIGHT

DYES	CLASSIFICATION (CONN, 1940)	WAVE-LENGTH OF LIGHT IN Å IN REGION OF MAXIMUM ABSORPTION	PERCENTAGE OF LIGHT ABSORBED BY DYES (2×10^{-5} M) IN REGION OF MAXIMUM		MAXIMUM REDUCTION IN AVERAGE REACTION-TIME (IN SECONDS)	CONCENTRATION OF DYES IN MOLES		FLUORESCENCE
			Absorption	Stimulating Efficiency (5,050 Å)		Optimum	Lethal	
Eosin	Fluorane derivative of xanthene	5,000	96.3	96.3	10.70	2×10^{-5}	1×10^{-4}	Strong
Rose bengal	Fluorane derivative of xanthene	5,300	100	92.0	11.04	2×10^{-5}	1×10^{-4}	Weak
Neutral red	Azin of the quinone-imine group	5,000	64.3	64.3	11.00	5×10^{-5}	1×10^{-4}	Weak
Methylene blue	Thiazin of the quinone-imine group	6,500	98.5	17.5	10.60	5×10^{-5}	1×10^{-4}	Very weak
Nile blue sulphate	Oxazin of the quinone-imine group	5,900	95.5	41.5	7.96	2×10^{-5}	5×10^{-5}	Very weak
Auramine O	Diphenyl methane	4,400	79.5	2.0	6.62	5×10^{-5}	2×10^{-4}	0
Orange G	Derivative of azobenzene	4,700	81.0	76.5	0	1×10^{-3}	5×10^{-4}	0
Brilliant green	Diamino triphenyl methane	6,200	100	37.5	0	1×10^{-5}	5×10^{-6}	0

Table 3 shows that the results obtained in the present work confirm this conclusion. This is particularly evident in those obtained with two of the four most effective dyes, namely, eosin and methylene blue. The results obtained with these two dyes are nearly the same, yet their molecular structures differ greatly, for methylene blue is in the quinone-imine group whereas eosin is a xanthene dye.

Wave length.—The reduction observed in the time required for reaction to light, caused by the effect of dyes on *Peranema*, must result from either an increase in the absorption of light by the photosensitive substance involved in the response, owing to the presence of the dyes in it, or an increase in the sensitivity of this substance caused by the dyes.

Shettles (1937) found that for motor response of *Peranema* in different regions of the spectrum, the stimulating efficiency increases rapidly to a maximum at 5,050 Å (as the wave length increases from 4,000 Å) and then decreases nearly to zero at 6,500 Å. Table 3 shows that in the region of maximum stimulating efficiency (5,050 Å) eosin, under the conditions given above, absorbs 96.3 per cent and methylene blue only 17.5 per cent, but that the effect on the reaction time is practically the same; the average reduction being 10.7 seconds in the one and 10.6 seconds in the other. The results obtained with rose bengal or neutral red and auramine O show practically the same thing.

These facts seem to prove that the observed reduction in reaction-time is not due to increase in the absorption of light by the photosensitive substance, owing to the presence of dyes in it, and that it therefore must be due to increase in its sensitivity caused by the dyes. They also show either that the effect on the sensitivity of the photosensitive substance of a given wave length of light (e.g., 5,050 Å) absorbed by different dyes differs greatly, or that the effect of each dye is correlated with the total quantity of light absorbed by it and is therefore independent of the wave length.

There is no support whatever for the proposition that various dyes are affected differently by a given wave length of light. According to the latter, the light absorbed by the dyes produces changes in them of such a nature that they cause increase in the sensitivity of the photosensitive substance. This proposition is in harmony with the conclusion of Straub (1904), Lippay (1929, 1930), Blum (1930), and others that oxidation of the dyes is involved in photodynamic action and also with the conclusion of Shettles (1937) that strychnine causes marked increase in the sensitivity of *Peranema* to light.

Toxicity.—Nearly all photodynamic actions result in injury of the substance involved. This does not, however, obtain for the increase in the sensitivity of *Peranema* produced by dyes; for, as Table 3 shows, orange G is much less toxic and brilliant green much more toxic than rose bengal and yet neither appears to have any effect on the motor response, whereas rose bengal has a very marked effect.

The decrease in sensitivity observed with the higher concentrations of the dyes and especially with brilliant green (Tables 2 and 3) is, however, doubtless correlated with toxicity.

The reciprocity law (IT = K).—Very few of the results obtained in the investigations concerning photodynamic action are in accord with the reciprocity law. Blum (1941) has considered this matter, and he concludes that this is due to "complicating factors." He says: "Since the only experiment in which such 'complicating factors' were ruled out shows good agreement with the reciprocity law, it is reasonable to assume that the underlying photochemical mechanism follows this simple relationship."

In the present experiments the amount of light necessary to evoke a motor response in *Peranema* can be calculated for various concentrations of dye solutions by taking the average of the absorption of light in the different regions of the spectrum for each concentration as a measure of intensity (*I*) and multiplying it by the time required to induce a response (Table 2). This was done for eosin, and the results obtained are presented in Table 4. This table shows that the absorption of light by eosin is 5.5 times as great in 1×10^{-5} M as it is in 1×10^{-6} M and 7.4 times as great in 2×10^{-5} M, and that the product of the light absorbed and the time required to induce a response (*IT*) increases from 2.5 in 1×10^{-6} M to 10.4 in 2×10^{-5} M.

Similar results were obtained by calculations for all the other dyes which increased the sensitivity of *Peranema*. These results are similar to those obtained by Lillie *et al.* (1935) in their observations on muscles. They show that the reciprocity law does not hold for the data presented. They, however, obviously do not invalidate Blum's conclusion presented above, for there doubtless are a number of processes between the absorption of light by the dyes and the motor responses involved.

Location of photodynamic action.—Lillie *et al.* (1935) maintain that the photodynamic action in muscles is primarily "a surface effect rather than one involving diffusion into the cell."

TABLE 4

DATA CONCERNING THE RECIPROCITY LAW IN REFERENCE TO RESPONSE OF *Peranema* TO LIGHT IN SOLUTIONS OF EOSIN

Molecular Concentration of Eosin	Relative Amount of Light Absorbed (I)	Reaction-Time of *Peranema* (T)	IT
2×10^{-5}......	7.4	1.4	10.4
1×10^{-5}......	5.5	1.4	7.7
1×10^{-6}......	1.0	2.5	2.5

Table 3 shows that the maximum effect on the reaction-time in *Peranema* is essentially the same for neutral red and rose bengal and also for methylene blue and eosin. However, neutral red and methylene blue diffuse readily through the entire cytoplasm in *Peranema*, whereas rose bengal and eosin are chiefly confined to the surface. This seems to show that photodynamic action in this organism is not closely correlated with the location of the dyes in it.

SUMMARY

1. Rose bengal, neutral red, eosin, and methylene blue have a great effect on the motor response of *Peranema* to light; Nile blue sulphate and auramine O have a moderate effect; brilliant green, a slight effect; and orange G, no effect.

2. The effect is closely correlated with the concentration of the dyes.

3. It is not specifically correlated with the structure of the molecules of the dyes, but it appears to be to some extent correlated with their fluorescence.

4. Light causes chemical changes in the dyes, after which they probably affect the motor response of *Peranema* to light by inducing changes in the photosensitive substance which make it more sensitive to light.

5. The photodynamic effect of dyes on the motor response of *Peranema* to light is not in accord with the reciprocity law.

6. The active dyes cause a very marked decrease in the variability of the motor response of *Peranema* to light.

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THE RELATION BETWEEN CONCENTRATION OF GROWTH-PROMOTING SUBSTANCE AND ITS EFFECT ON GROWTH IN CHILOMONAS PARAMECIUM[1]

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ROBERTSON (1922, 1923, 1924) was the first to suggest the production of a growth-accelerating substance by protozoans. Since that time numerous investigations have been made concerning the possibility of his "allelocatalytic effect." Most of the workers in this field disagree with Robertson, but some have obtained results in full or partial agreement (see discussion by Allee, 1931 and 1934, or Mast and Pace, 1938). Not all the work on this subject has been carried out with cultures under properly controlled conditions, chiefly because of bacterial contamination.

Chilomonas paramecium has been used extensively in cellular studies by numerous investigators. It was first cultured in pure, sterile organic solution (acetate-ammonium solution), as well as in inorganic solution, by Mast and Pace (1933) and proved to be an excellent organism for the investigations which followed (Mast and Pace, 1935–42; Smith, 1940; Hutchens, 1941; Bowen, 1940).

Using adequately controlled techniques, Mast and Pace (1938) reported the production of a growth-promoting[2] substance by *Chilomonas*. They claim that a single substance is produced by this organism, which accelerates growth in low concentration but retards or inhibits growth in high concentration. They have also found that rate of reproduction varies with the amount of growth substance present in the protoplasm of the individual. The rate of reproduction, when organisms are put into fresh acetate-ammonium solutions, is slowest in chilomonads taken from old parent cultures. Mast and Pace (1938) suggest that such organisms contain a

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[2] Hereafter the growth-promoting substance will be referred to as "growth substance."

high concentration of growth substance in their protoplasm. The most rapid division occurs in the cultures to which chilomonads from a young parent culture are added; these chilomonads are said to have a low concentration of growth substance in their protoplasm.

The rate of reproduction in culture fluid containing this substance in a given concentration varies with the age of the parent culture from which the chilomonads are taken; i.e., the older the culture, the greater the concentration of growth substance and the slower the rate of reproduction (Mast and Pace, 1938).

In recent investigations Kidder (1941) finds that the ciliate *Tetrahymena geleii* may elaborate two substances—one an accelerator, produced during early growth phases, the other an inhibitor. He worked under aseptic conditions, as did Marbarger (1943), who claims that *Colpidium striatum* (this is really the same species [*T. geleii*] as was used by Kidder) produces a growth substance which accelerates or retards the rate of reproduction depending on the concentration.

The number of protozoan species which can be cultured under aseptic conditions are few; but in most of those that can be cultured, the production of growth substances has been clearly demonstrated. It is imperative that aseptic conditions prevail in order that the growth-promoting powers of single-species cultures in bacteria-free media may be critically investigated.

The following experiments were carried out with the view of extending some of the observations of Mast and Pace.

MATERIAL AND METHODS

The chilomonads used in these experiments were from the Mast and Pace strain that has been cultured over a period of 12 years in acetate-ammonium solution. The solution was modified only slightly during these years; and for the experiments reported here it consisted of CH_3COONa, 125 mg.; NH_4Cl, 46 mg.; $(NH_4)_2SO_4$, 10 mg.; K_2HPO_4, 20 mg.; $CaCl_2$, 1 mg.; $MgCl_2$, 1 mg.; triple-distilled water, 100 ml. The salts used were Kahlbaum's "purest."

The relative condition of the chilomonads with respect to age and concentration of growth substance was known, approximately, throughout the experiments. This knowledge is highly important, as results are sometimes affected considerably by these factors.

The division rate of chilomonads with different concentrations of growth substance in their protoplasm was recorded every hour for a period of 60–80 hours. This time is sufficient to cover the growth cycle of these organisms in the volumes of solutions used.

RESULTS

Experiment A.—Five hundred chilomonads from a known parent culture (started from one chilomonad which was added to 0.1 ml. of the acetate-ammonium solution and left 4 days) were put into 0.1 ml. of the acetate-ammonium solution in a depression on a slide which had been previously sterilized in a Petri dish. This was then placed in a constant-temperature chamber. The culture remained in the chamber for 6 days (24.5° ± 0.2° C.) and is therefore referred to as the one containing "old" chilomonads (see Table 1). Four days later, five hundred chilomonads (same condition as above) were put into 0.1 ml. of the acetate-ammonium solution; and at the same time one chilomonad was put into 0.1 ml. of the solution in depressions on slides in Petri dishes, which were then placed in the constant-temperature chamber. The organisms later obtained from these cultures are referred to as th

TABLE 1

THE RELATION BETWEEN AGE OF PARENT CULTURE AND GROWTH IN *Chilomonas paramecium*

Age of Chilomonads	Average Time (in Hours with Standard Errors) for Chilomonads in 24 Isolation Cultures												
	To First Division	First to Second Division	Second to Third Division	Third to Fourth Division	Fourth to Fifth Division	Fifth to Sixth Division	Sixth to Seventh Division	Seventh to Eighth Division	Eighth to Ninth Division	Ninth to Tenth Division	Tenth to Eleventh Division	Eleventh to Twelfth Division	Twelfth to Thirteenth Division
	Experiment A												
Young..........	6.16±0.51	5.08±0.18	4.44±0.11	5.75±0.01	6.03±0.11	6.90±0.11	8.98±0.06	8.45±0.003	9.62±0.01	....	....	....	....
Medium-aged....	7.20±0.52	4.40±0.11	4.75±0.08	6.10±0.12	6.67±0.11	7.15±0.05	7.31±0.05	8.08±0.04	9.23±0.03	....	....	....	....
Old............	10.75±0.68	4.64±0.16	5.50±0.12	5.88±0.13	6.46±0.10	7.58±0.04	9.12±0.04	10.13±0.06	....	....	....	....	....
	Experiment B												
Young..........	6.33±0.20	6.33±0.03	6.50±0.18	5.25±0.18	4.50±0.13	4.41±0.18	5.00±0.21	5.66±0.15	6.58±0.24	6.08±0.15	6.66±0.14	6.75±0.28	7.42±0.16
Medium-aged....	7.50±0.25	5.91±0.13	5.59±0.16	5.08±0.15	4.33±0.14	4.16±0.15	4.41±0.14	5.25±0.11	6.00±0.19	6.36±0.22	6.72±0.15	7.63±0.32	8.20±0.21
Old............	9.83±0.34	6.16±0.22	5.16±0.13	4.16±0.17	4.33±0.03	4.75±0.11	5.16±0.17	5.66±0.16	6.08±0.21	6.81±0.22	6.63±0.20	7.36±0.34	8.14±0.28
	Experiment C												
Young..........	6.42±0.28	6.08±0.24	6.42±0.14	5.58±0.20	5.92±0.21	5.83±0.16	6.17±0.20	6.08±0.27	5.75±0.17	6.00±0.18	6.00±0.22	5.92±0.14	6.21±0.31
Medium-aged....	7.83±0.43	5.00±0.28	4.58±0.20	5.17±0.18	5.83±0.26	5.42±0.17	5.92±0.12	6.00±0.28	6.33±0.16	5.83±0.22	6.00±0.18	6.10±0.22	6.18±0.28
Old............	11.18±0.70	6.50±0.58	4.90±0.28	4.60±0.37	5.40±0.20	6.20±0.21	5.90±0.25	6.20±0.26	6.30±0.14	5.90±0.22	6.10±0.20	6.20±0.21	5.92±0.17
	Experiment D (Average Time [in Hours with Standard Errors] for 5 chilomonads in each of 24 cultures, 10 in Each of 24, and 100 in Each of 12, Respectively)												
Old (5 chilomonads)..........	11.25±0.73	5.75±0.33	6.50±0.18	7.25±0.21	10.35±0.16	....	....	....	....	....	....	....	....
Old (10 chilomonads)..........	11.72±0.82	5.52±0.41	6.94±0.23	8.75±0.17	11.50±0.12	....	....	....	....	....	....	....	....
Old (100 chilomonads)..........	15.30±0.02	....	....	....	....	....	....	....	....	....	....	....	....

"medium-aged" and "young" chilomonads, respectively. Two days later, twenty-four chilomonads were taken from each of these three cultures, immediately following division; and one individual was introduced into each of seventy-two depressions on slides containing 0.025 ml. of acetate-ammonium solution (they were first washed in sterile acetate-ammonium solution, as described by Mast and Pace, 1938; the same is true for all the other chilomonads used in these experiments). Water was put in the bottom of each Petri dish containing the slides, and these were then placed in the temperature chamber at 24.5° ± 0.2° C. They were taken out at the end of each hour, and the time required for each division noted. This was repeated hourly, which allowed much less than 1 minute for examination of each culture. However, in this small volume of solution the whole area can be scanned, usually at a single glance, with the stereoscopic microscope. This was true for Experiments B and C, but in the latter part of Experiment A and in Experiment D more than 2 hours were required in some cases for a hurried count of all the cultures. A close approximation of rate of reproduction was obtained by this means even in the very crowded cultures.

The temperature of the room was approximately the same as that of the constant-temperature chamber. Distilled water was added whenever evaporation occurred, to maintain a constant volume. This was accomplished by means of capillary pipettes, which were graduated to hold 0.025 ml. The solution (with the chilomonads in it) was drawn into the pipette and then made up to 0.025 ml. with redistilled water. The solutions and chilomonads in all the other depressions, even though evaporation had not occurred in them, were treated in this same manner. They were then returned to the slides. Very little evaporation occurred, evidently because of the extreme care taken to insure a saturated vapor surrounding the solutions. The hydrogen-ion concentration for this and later experiments was kept at pH 6.8.

In this experiment, after counting and noting the time of the divisions, the Petri dishes were again placed in the constant-temperature chamber until the next hour. The experiment was carried out until the ninth division and required constant observation for over 60 hours.

The results are presented in Table 1, Experiment A, and in Figure 1. In chilomonads taken from the "old" parent culture the average time to first division was 10.75± hours. This is 4.5 hours longer than the average time to first division in the chilomonads taken from the "young" parent culture. The chilomonads from the "medium-aged" parent culture required, on the average, about 1 hour longer to first division than those from the "young" parent culture.

During the second division the chilomonads from the "old" parent culture required only 4.64 hours to divide, or approximately 6 hours less than for the first division. From the third division on, there is a gradual increase in the time required for division. The conditions reached by the end of the eighth and ninth divisions are similar to the conditions found in chilomonads taken from "old" parent cultures. The relationship is clearly shown in Figure 1. This condition occurs when the chilomonads are allowed to collect in the culture solution and represents the growth of a population as it might occur in a natural habitat.

Experiment B.—In this experiment the "young," "medium-aged," and "old" chilomonads were obtained in the same manner as those of Experiment A. Twenty-four chilomonads were taken from each of the three different types of cultures, and one was put into each of seventy-two depressions (containing 0.025 ml. of culture fluid) on slides. The very same procedure was followed as in Experiment A except that after each division one of the chilomonads was drawn from the solution and discarded. Thus, there was never more than one chilomonad in each depression except immediately after division. These cultures were observed carefully and constantly until the thirteenth division (or over 70–80 hours). It takes a considerably longer time for a retardation in division even in this small volume than it took in Experiment A, where the chilomonads were never discarded but were allowed to collect until the end of the experiment.

The results are presented in Table 1, Experiment B, and in Figure 2. The time to first division in "young," "medium-aged," and "old" chilomonads corresponds very closely to the time for the comparable types in Experiment A (as well as in the following experiment, C). The difference lies in the fact that an optimum condition is found at the fifth or sixth division rather than at the second or third, as in Experiment A.

Experiment C.—The same procedure was followed as in Experiment B except that, after each division, one of the chilomonads was transferred to fresh solution. Three types of chilomonads were again used, and twenty-four of each type added to the depression slide cultures (seventy-two in all).

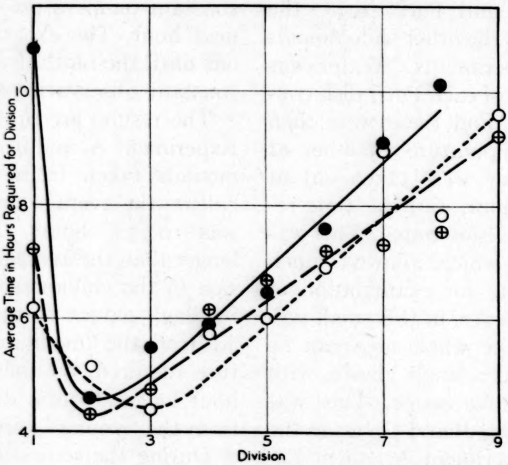

FIG. 1.—The growth cycle of *Chilomonas paramecium* showing the relationship between the concentration of growth substance in the protoplasm and rate of reproduction in 0.025 ml. of acetate-ammonium solution. Temperature, 24.5° ± 0.2° C.; pH, 6.8. Each point represents the average time to division in twenty-four cultures, each started with one chilomonad. ○, chilomonads from young parent culture; ⊕, from medium-aged culture; ●, from old culture.

As shown by the results (Table 1, Expt. C, and Fig. 3), there is an acceleration after the first or second division. A more or less constant rate is found after the fourth division. This rate is not optimum. Evidently, because of the constant transfer, the chilomonads never become "old."

Experiment D.—In this experiment only chilomonads from an "old" parent culture were used. They were carried

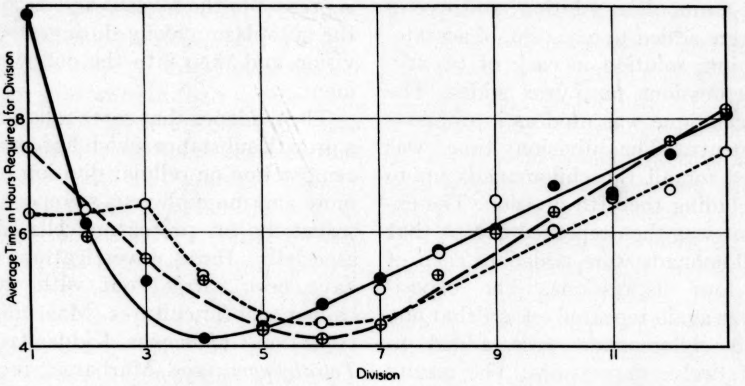

FIG. 2.—The relation between the concentration of growth substance in *Chilomonas* and rate of reproduction in 0.025 ml. of solution when one of the chilomonads is removed and discarded immediately after each division. Temperature, 24.5° ± 0.2° C.; pH, 6.8. Each point represents the average time to division for twenty-four chilomonads. ○, chilomonads from young parent culture; ⊕, from medium-aged culture; ●, from old culture.

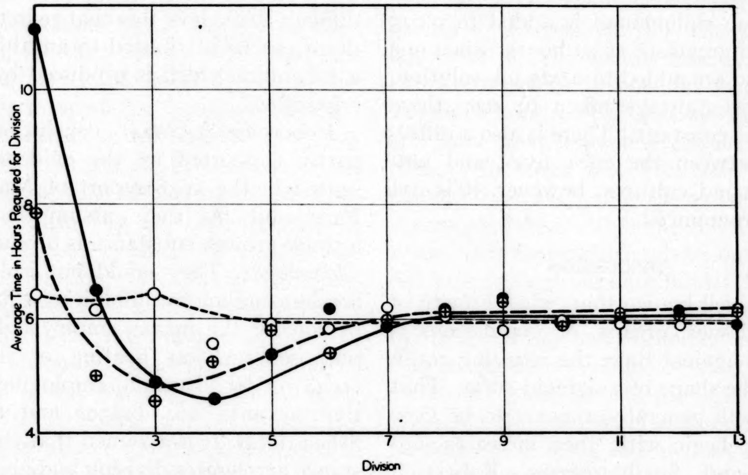

FIG. 3.—The relation between the concentration of growth substance in *Chilomonas* and the rate of reproduction in 0.025 ml. of solution when, immediately following division, one chilomonad is transferred to fresh solution and the other discarded with the old solution. Temperature, 24.5° ± 0.2° C.; pH, 6.8. Each point represents the average time to division for twenty-four chilomonads. ○, chilomonads from young parent culture; ⊕, from medium-aged parent culture; ●, from old culture.

through eight washings in fresh, sterile acetate-ammonium solution; and five of them were added to 0.025 ml. of acetate-ammonium solution in each of twenty-four depressions on pyrex slides. The same technique was used as in previous experiments. The division time was recorded for all the chilomonads up to and including the fifth division. The experiment was then repeated except that ten chilomonads were added to each of twenty-four depressions. The experiment was again repeated except that one hundred chilomonads were added to each of twelve depressions. The results are presented in Table 1.

It is evident that the lag phase—at least in this volume of solution—depends, to a certain extent, on the number of chilomonads introduced. That is, the lag phase is shortest (10.75 hours) when only one chilomonad is added to 0.025 ml. and greatest (15.30 hours) when one hundred are added to 0.025-ml. solution. (Care was always taken to keep these volumes constant.) There is also a difference between the one-, five-, and ten-chilomonad cultures; however, it is not very pronounced.

DISCUSSION

It is well known that, when growth of cells, tissues, organs, or populations is plotted against time, the resulting graph takes the shape of a sigmoid curve. That is, growth generally appears to be very slow, to begin with, then increases suddenly and finally ceases. Robertson (1923) assumes that the position of greatest slope on the curve represents the optimum at which autocatalysis is taking place. He contends that, as cells divide, there is naturally a greater increase in the concentration of the autocatalyst, which, when it becomes too great, retards growth and finally inhibits it altogether. He suggests that the substance which accelerates cellular division is present in the nucleus and escapes into the cytoplasm chiefly during nuclear division and then into the outer environment.

The evidence that some cells elaborate a growth substance which has an accelerating action on cellular division becomes more and more obvious as reports of observations on protozoan cells increase, especially those investigations which have been carried out with well-controlled aseptic cultures. Mast and Pace (1938) on *Chilomonas*, Kidder (1941) on *Tetrahymena*, and Marbarger (1943) on *C. striatum* (= *T. geleii*), all worked under aseptic conditions and found that the media used in their respective investigations, which have supported growth of these organisms for a time, possess accelerative or inhibitory qualities. It is difficult to see how this change in the medium can be attributed to anything but a substance which is produced by these organisms.

Robertson's (1924) conclusions are partly supported by the evidence presented in the 1938 report of Mast and Pace and by their assumption that a single growth substance is produced by *Chilomonas*. They could not isolate an accelerating and an inhibiting substance by any of the means employed for the purpose, such as heating or diluting (1938) or by filtration, employing selective solvents, etc. (1939*b* and unpublished data). It is assumed that this substance accelerates division and growth in low concentration and retards or inhibits in high concentration. Thus, in organisms taken from "old" parent cultures, the substance is assumed to be in such high concentration that, when these organisms are placed in fresh solutions, it takes considerable time for the substance to diffuse out. After sufficient exposure to fresh solution, a lowering of the concentration is reached within the cell; and the nucleus and cell divide. According to this hypothesis, "young" chilomonads have a low concentration of growth substance in their protoplasm, while "medium-aged" chilomonads have a concentration somewhere between that of the "old" and the "young." Usually the shortest time between division is found at the second or third division after a cell or organism is placed in a fresh solution (see Table 1 and Figs. 1, 2, and 3). At this time the diffusion of growth substance out of the cell results in an optimum concentration within the cell. If diffusion goes on at a rather rapid rate, a suboptimum concentration may be reached, which, if low enough, results in a retardation, if not an actual inhibition, of division. Such a condition is found in Experiment C. The chilomonads were transferred as rapidly as they divided, so that the growth substance could not collect in the surrounding solution. Because of the constant transfer, the concentration of growth substance in the solution and also in the protoplasm of the organisms becomes suboptimum. This condition is also found when a single organism is placed in a large volume of solution. If such an organism grows and divides at all, it does so at a much reduced rate (Mast and Pace, 1938). However, when the volume of solution is not too great, we find that, soon after reaching an optimum, the growth substance gradually increases in concentration in the surrounding medium (and consequently within the cell) until the level becomes high enough again to retard and inhibit division.

Robertson (1923) found that cell proximity does not result in a mutual acceleration during the lag phase. When one cell is placed in 0.8 ml. of solution in a depression, and likewise two cells in 0.8 ml. of solution in another depression, the latter shows an average lag period as great as, if not greater than, that found in the single individual. He contends that there is no greater acceleration in the one than in the other, because the growth substance is not distributed until the nucleus divides; and so an optimum condition is not reached until after the first or second division.

The production of growth substance, however, need not be limited to the nucleus. As previously stated, the assumption is that in "old" cultures relatively great quantities of growth substance are present. A culture becomes old as the population increases. In this way the quantity of any substance produced within the cells will increase in the surrounding environment and naturally within the cells themselves. Further support for this contention is found in the results of Experiment D. The average lag phase in chilomonads taken from an old parent culture is considerably greater when one hundred chilomonads are present than when only one chilomonad is present in 0.025 ml. This can be explained by considering the growth substance produced within the cell as a diffusible substance which passes out of the cell into the surrounding fluid. This diffusion is much more rapid for a single cell than for any one of the one hundred cells in the same volume of solution. As the substance collects in the surrounding solution, the process of diffusion is retarded. Since there is much more substance thrown out by one hundred cells, diffusion becomes slower for each individual cell in these cultures than for each cell in the one-cell cultures. The time to first division is therefore longer in the one-hundred-cell cultures because the growth substance requires a longer time to fall to an optimum concentration within each cell.

The lag period is defined by Robertson

(1923) as the period preceding the first cell division and by Kidder as "the period elapsing between the inoculation and the beginning of logarithmic growth" (1941). This period is especially noticeable when the cell is taken from an "old" parent culture and placed in fresh solution. The true lag, in so far as a lag in rate of reproduction is concerned, very seldom, if ever, goes beyond this period, although many investigators look upon the lag period as a phase of growth represented by that portion of the growth curve just preceding the steepest rise or the beginning of "logarithmic growth." The position indicating most rapid division, however, lies within this lag period, especially if comparatively few cells are introduced into a culture. Robertson (1923) has indicated that, if organisms are transferred from a rapidly growing culture (i.e., one in the so-called "logarithmic phase" of the growth cycle) to fresh solution, no lag period is evident. This has been verified by numerous investigators. As brought out previously in this discussion, chilomonads can be placed in fresh solution immediately following the second or third division with no indication of a lag phase. Conditions comparable with those reported by Robertson and others are found in Experiment C, in which the chilomonads were transferred to fresh solution as rapidly as they divided and in which, after the first division, no lag was apparent. Evidently, in organisms from a rapidly growing culture or in those which are transferred to fresh solution after each division, the growth substance becomes optimum (or suboptimum) in concentration by diffusion into the surrounding solution, and it does not go above this concentration unless the organisms remain in the solution without transfer.

The terminology of the bacteriologist does not always coincide with that of investigators in other fields, and for this reason much confusion is found in the literature concerning lag. Buchanan (1918), in reference to bacterial growth, speaks of an "initial stationary phase" and a "lag phase." This lag phase is also referred to as the "positive growth-acceleration phase." Surely the growth processes of bacterial, protozoan, and metazoan cells are not so dissimilar that the term "lag" should apply to different processes in these various types. The term should be reserved, at least in protozoan work, for that period of "rejuvenescence" which begins at the time of introduction of a cell (usually an "old" cell) into fresh culture solution and ends at the first division or soon after.

Richards, in a very interesting review on the growth of Protozoa, states that "the sigmoid nature of the growth curve is the inevitable result of the regular geometrical increase during the time that the environment is favorable, and the slowing of this increase when the environment becomes unfavorable as a result of the growth in it (decrease of foodstuffs and accumulation of excretion products)" (1940). This view is not held so generally as it was a few years ago. Undoubtedly, these factors play some part in the normal regulation of growth, but they are not the chief reasons for growth retardation in *Paramecium* or *Chilomonas*.

Robertson (1923) grew a second population of *Paramecium* in a culture solution after filtering off the first population (or destroying the paramecia by heating and then separating by means of a centrifuge) from an old hay-infusion culture. Sufficient foodstuffs seemed to be present, and the excretory products seemed to have no effect on growth. However, Woodruff (1911), Petersen (1929), Di Tomo (1932), Beers (1933), and Jahn

(1934) claimed that excretory products retarded division in the forms with which they worked. Phelps (1936) on *Glaucoma*, Taylor and Strickland (1938) on *Colpoda*, and Johnson and Hardin (1938) on *Paramecium* found that excretion products have very little effect on growth. Mast and Pace (1939*a*) and Pace (1941) have shown that culture solutions can support a second population of chilomonads (or even a third), without the addition of foodstuffs, after growth has ceased in the first population, or after it has died out, if the solution is heated to 99.5° C. for from 0.5 to 2 hours. The chilomonads can be taken out before heating by centrifuging. The contention is that the growth substance, which in high concentration inhibits further growth of the chilomonads, is destroyed by heating and that the food is not depleted, nor the excretory products too highly concentrated, since a second population is produced upon the addition of chilomonads. This second population does not reach the maximum number of the first; but very good growth is obtained, which is sufficient to establish the fact that growth does occur without the addition of food or the removal of wastes, except those destroyed by heating. All the evidence seems to point to the possibility that in nature this substance may act as a naturally occurring regulator for growth in these organisms and that the extent of populations may be controlled by the retardation produced by the substance in high concentrations. Perhaps a definite ratio must exist between the concentration inside and outside the cell in order that conditions become optimum, and any change in this ratio may result in a decrease in rate of reproduction.

The amount of growth substance formed by chilomonads in unit time seems to be rather constant. In comparing the results of Experiments A and B, Table 1, obtained for chilomonads with "low" concentration of growth substance in their protoplasm, it will be noted that in Experiment A, the optimum concentration occurred during the third division, while in Experiment B it occurred during the sixth division. However, the chilomonads were never discarded after division in Experiment A, so that up to the third division there were, respectively, one, two, and four cells in 0.025-ml. solution, while in Experiment B there was never more than one cell present because after each division one cell was discarded. If the "chilomonad-hours" necessary to produce an optimum concentration of growth substance is calculated for each experiment, the results are surprisingly similar for both; 34.08 chilomonad-hours in Experiment A and 33.32 chilomonad-hours in Experiment B are required to produce an optimum concentration. A fully optimum condition was never obtained in the five- and ten-chilomonad cultures of Experiment D, so that a comparison with the results of Experiments A and B is not feasible.

SUMMARY

1. A strain of *Ch. paramecium* has been grown for a period of 12 years in a solution containing CH_3COONa, NH_4Cl, K_2HPO_4, $MgSO_4$, and $CaCl_2$.

2. Further evidence is presented to show that a growth substance is elaborated by *Chilomonas* which in low concentration accelerates but which may accumulate in the protoplasm and cause a retardation or inhibition in the rate of reproduction.

3. The growth substance apparently diffuses constantly into the external environment as long as the proper concentration gradient exists. If the concentration in the environment already exceeds

that of the cell, there is a diffusion into the cell.

4. When a single chilomonad is transferred to fresh solution after each division, a suboptimum concentration of growth substance is produced in the protoplasm because the substance diffuses out at a rate too rapid for it to collect in the cell in optimum concentration.

5. The quantity of growth substance present in solution and in the protoplasm of chilomonads depends upon the age of the organisms put into the solution. Likewise, the lag period which is evident when chilomonads are added to fresh solutions depends upon the age of the organisms.

6. The greater the number of chilomonads introduced into a given volume of solution (0.025 ml.), the more evident is the lag phase. This is assumed to depend upon the rate of diffusion of growth substance out of the cells.

7. The lag phase is the result of a period of "rejuvenescence" and is the time required for diffusion of growth substance out of the cell until the concentration within becomes low enough to permit division.

8. Evidently, the production and diffusion of growth substance is fairly constant. In 0.025 ml. of acetate-ammonium solution an optimum concentration of growth substance is produced in 33–35 "chilomonad-hours" if the chilomonads employed have a low initial concentration of growth substance in their protoplasm.

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SOME ASPECTS OF THE BIOLOGICAL ACTION OF X-RAYS ON COCK SPERMATOZOA

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BIOLOGICAL action of short-wave radiation on the functional activity of gametes has been the subject of extensive studies since the turn of this century. Bohn's observations in 1903 showed that, when the spermatozoa of *Strongylocentrotus lividus*, a sea urchin, had been exposed for some time to radium emanations and were then allowed to fertilize ova, some of the ova developed parthenogenetically. He did not pursue the matter further. It was left to Oscar Hertwig and his group to study this interesting aspect of the problem in greater detail. Hertwig (1911) found that, if spermatozoa of *Rana viridis* and *R. fusca* are left exposed to radium for a number of hours, the proportion of malformed tadpoles fathered by such spermatozoa is smaller than when the male gametes are only lightly irradiated. Hertwig explained this condition by assuming that the prolonged exposure of spermatozoa to radium emanations completely disorganizes their chromatin material. When the spermatozoa are brought into contact with mature ova, they activate development without contributing any hereditary material to the zygote.

This ingenious explanation appeared to have received confirmation two years later, when Hertwig (1913) succeeded in

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fertilizing anuran eggs of one species with the irradiated spermatozoa of another. As he had anticipated, the tadpoles from such a cross were all of maternal type.

Other investigators soon corroborated the occurrence of this phenomenon in other forms: G. Hertwig (1912), sea urchin; Oppermann (1913), trout; P. Hertwig (1916), triton; Packard (1914, 1918), marine worms.

A somewhat similar situation was observed by Dalcq (1930) when he exposed spermatozoa of *R. fusca* to ultra-violet rays. A year later, however, Dalcq and Simon (1931) reported their failure to obtain fertile eggs when the spermatozoa of the same species of frogs were pretreated with X-rays in excess of 3,000 roentgen units. The "Hertwig phenomenon" was absent. Rugh (1939) carried out an intensive study of the problem with X-rays, using spermatozoa and eggs of *R. pipiens*. Contrary to the results of Dalcq and Simon, Rugh corroborated, in broad general terms, Hertwig's original observations subscribing to his interpretation of the phenomenon. The following year Rugh and Exner (1940) carried out crossbreeding tests between the X-rayed spermatozoa of bullfrog and leopard-frog ova. The results added further weight to their earlier observations.

From this brief introductory review of relevant literature it will be seen that, although considerable data have accumulated on this peculiar quality of shortwave irradiation, all the forms studied belonged to invertebrates or to lower vertebrates. The recently reported work of Henson (1942) is the only investigation known to the present writer which, in part at least, deals with the topic under discussion but which uses a higher form (albino rat) as the experimental animal.

From both practical and theoretical considerations it was deemed interesting to extend such observations to the domestic fowl, *Gallus gallus*. If the X-ray "neutralized" spermatozoa are able to induce gynogenetic development in certain forms, would it not be possible to achieve similar results on birds? Present-day breeding practices in the poultry industry are directed to the purification of the existing strains for desirable characteristics. However, the methods used, in common with all animal improvement work, are incapable of achieving this goal. The fact that each parent, itself heterozygous (at least for a number of genes), contributes to the genic makeup of the offspring works against homozygosity. A gynogenetic individual, on the other hand, stands a good chance of being homozygous by virtue of the fact that only one parent—in this case the mother—provides it with its complement of hereditary material. If the matter is not complicated by the suppression of, or the delay in, the expulsion of the polar bodies, a "homozygous" individual is produced. If this reasoning is projected to chickens, in which the female sex is heterogametic, successful application of this principle should be formed to result in "homozygous" male zygotes. If such zygotes are viable, it would mean that such birds, selected for desirable qualities, could be used as sires in order to increase the homozygosity of the population. The immediate aim of the study was to determine the feasibility of such a scheme in poultry-breeding work. That this need not have been an impossible feat was suggested by the work of Pincus (1939), who obtained fertile rabbits (females) from parthenogenetically developed ova.

EFFECT OF X-RAY ON THE FERTILIZING CAPACITY AND VIABILITY OF SPERMATOZOA

FERTILITY STUDIES

The discovery of practical methods of obtaining cock semen and of artificial insemination in the fowl (Munro, 1935; Burrows and Quinn, 1935; Quinn and Burrows, 1935) added valuable techniques to the list of available experimental tools. The semen thus obtained can be easily subjected to various treatments. In the first series of studies reported here the basic treatment consisted of irradiating the cock semen with various doses of soft X-rays, followed by artificial insemination. The latter technique allows the observer to exercise complete control over the volume of the semen allotted to each bird, identity of the hens inseminated, and the time of insemination.

The cock semen lends itself well to this type of work, for a number of reasons. One of these is the ease with which the semen is obtained. The relatively large volume of the ejaculate is another factor in its favor. Absence of the coagulating gland in the genital system of gallinacious birds is also an obvious advantage.

The first group of experiments was begun at the Poultry Division in August, 1940, and was concerned primarily with the establishment of the biotic (physiological) dose range for the Roentgen radiation of cock spermatozoa.

Two healthy Barred Plymouth Rock males were selected to provide the semen. As males vary in their capacity to produce satisfactory ejaculates (from the standpoint of ease of ejaculation, volume of the ejaculate, and its nature), preliminary tests were carried out to select the two birds which ranked best in this respect among a large flock of young, mature males. In the tests of this series, in contrast to later studies, every sample of semen from each male was treated individually. On ejaculation the specimen was divided into two equal parts—one to be irradiated, the other to serve as control.

Irradiation energy was provided by a General Electric portable X-ray machine, operated at 65 kv. and 4 ma. The target distance was 3 cm. With the selector set at L 12, the machine delivered 924 r of soft X-rays per minute. Before radiation, both halves of the sperm sample—i.e., one slated for irradiation and one for the control lot—were transferred into small, specially made celluloid receptacles. The vessels were flat and shallow, so that in no case did the sperm suspension exceed 2 mm. in depth. This precaution was necessary in order to avoid, as much as possible, the effects of shielding on subsequent results. As will be pointed out later, even with this precaution it is not at all certain that the situation was completely controlled.

The doses ranged from 231 r to 36,800 r. In other words, the highest dose secured in the first series represented 40 minutes of irradiation. Because of the manufacturer's instructions, no single exposure lasted more than 3 minutes (2,772 r). Higher doses were obtained by split irradiations, with 15-minute intervals between each exposure. During such intervals the irradiated sample was removed and examined for motility of its spermatozoa. Parallel examinations were made of control specimens. If there appeared any undue decrease in motility in either sample, the experiment was repeated with fresh semen. It might be stated here that on only three occasions was it found necessary to resort to fresh specimens.

The technique of split irradiation was adopted as a matter of necessity rather than of choice. Its particular disadvantage lay in the fact that the treatment period in some cases was extended over several hours. Literature indicates that if the interexposure periods are not unduly long (not over 1 hour) the efficiency of such exposures compares favorably with the continuous form of radiation (Coutard, 1922, 1932; Warren and Whipple, 1923; Dempster, 1941; Kaufmann, 1941).

Following irradiation, both lots of semen were then inseminated into an equal number of Single Comb White Leghorn pullets of comparable age and breeding. All birds were kept in the same pen. As each male's single ejaculate was found to be adequate for four hens, eight females in all were inseminated on any one day. To assure adequate and continual supply of semen from the two males, ejaculates were collected on alternate days only. This made it possible to secure at least 0.5 cc. per male per ejaculation. Each hen received 0.1 cc. of semen, or about double the amount found necessary at this laboratory to assure good fertility.

The cross selected—Barred Rock males and White Leghorn females—produces cream-colored chicks with a few scattered black specks. The specks are due to the heterozygous condition of the color-inhibitor gene (*I*) in the F_1 generation. The presence of flecking was, therefore, a practical way of checking on the participation of the paternal chromatin material (supplying the recessive allelomorph to *I*) in zygotic development. As this point was of cardinal importance in these tests, all chicks, whether hatched or embryos dead in shell (if their development had gone sufficiently far), were invariably closely examined for the presence of flecking.

Eggs from hens thus "bred" were saved, beginning with the second day following insemination. O. K. Moore and Byerly (1942) have found that maximum fertility in artificially inseminated hens is obtained on the third or fourth day after insemination. Fertility then falls off rapidly. The bulk of the data in this series is based on eggs laid during the first 10 days, counting as day 1 the second day following insemination. This was done because in a laying hen an egg is likely to be present in the oviduct at the time of insemination. Such an egg, of course, will not be fertile. All eggs were "set" daily in an electric incubator. The first check on fertility was carried out 24 hours later. Doubtful cases were left in, together with the eggs which were classified definitely as "fertile." The former were re-examined the following day and, if found to be infertile, were removed. The fertile eggs also were examined daily as a matter of course, to provide some information on the rate of embryonic development in different groups. Moreover, these daily tests provided a good check on embryonic lethality.

Macroscopic appearance of the blastodisk was the usual criterion relied upon in identifying fertile ova. The ability of the observer to classify the eggs on the basis of the gross appearance of the blastodisk was tested by making histological preparations of a number of ova. As a preliminary experiment, a group of ova, both from mated and unmated pens, were studied histologically. This was done to compare the histological appearance of blastodisks known never to have started development with the appearance of those in which true segmentation was temporarily arrested following oviposition. Results of these comparative studies justified placing reliance on the gross method of segregation.

Rugh (1939) found that, when he irradiated frog spermatozoa with X-ray doses as high as 50,000 r, there was no diminution in the fertilizing capacity of such gametes. In the present investigation the cock spermatozoa were found to be extremely sensitive to Roentgen radiation. The data are summarized in Figure 1 and Table 1.

It will be seen that there appears to be an inverse type of relationship between the intensity of radiation and the subsequent fertilizing capacity of cock spermatozoa. This relationship is fairly simple. The coefficient of correlation between the two variables was calculated to be -0.9631, a condition best demonstrated by a straight line. The equation for such a regression line was found to be $25-0.3268\ y$. In calculating this equation, the values representing doses were coded: the value of 1 was substituted for 231 r, value of 2 for 462 r, and so on.

One way in which these results could be accounted for was to presume that the radiation treatment reduces, speaking anthropomorphically, the stamina of the radiated spermatozoa. The latter are then unable to reach the upper end of the oviduct to affect fertilization.

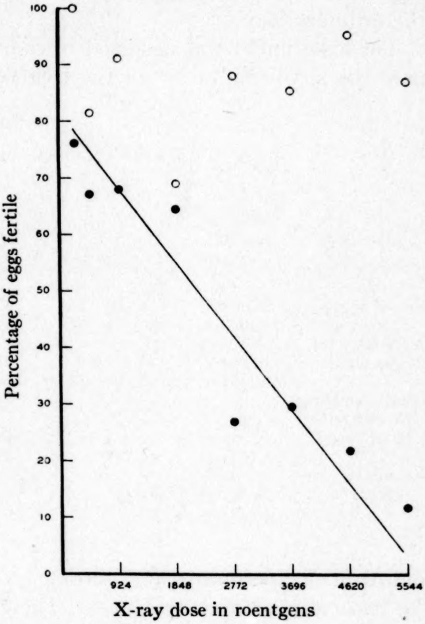

FIG. 1.—Effect of X-rays on fertility. ○ = control; ● = experimental.

It has been shown (Crew, 1926; Warren and Kilpatrick, 1929) that the process of fertilization in the hen takes place shortly after ovulation, when the ovum is still in the upper reaches of the oviduct. In the frog (*R. pipiens*) fertilization is, of course, of the external type. If, then, the "stamina" of the radiated spermatozoa is reduced, might it not be possible that the apparent immunity of the frog spermatozoa to X-rays (in so far as the fertilizing capacity is concerned) and the extreme sensitiveness of the cock spermatozoa are really due to different factors associated with sperm behavior? To test this possibility, intraperitoneal insemination was resorted to. In this way the cock spermatozoa are brought into almost immediate contact with the ova, simulating, to some extent at least, the external type of fertilization. To accomplish intraperitoneal insemination, a simple operation is required. The bird is anesthetized with Nembutal (about 25 mg. per kilogram of body weight) via the radial vein. The anesthetized bird is laid on its right side, with the legs pulled caudally to expose its left flank in the region of the sixth and seventh vertebral ribs. After the area is plucked, a small incision is made, about 3 cm. long, between the two ribs. The cut, which penetrates the body wall, opens into the peritoneal cavity, exposing the follicular mass of the left ovary. In most cases it may be found necessary to cut through the dorsal ligament of the oviduct to reach the ovary. The exposed gonad is then "irrigated" with the undiluted semen by means of the ordinary 1-cc. tuberculin syringe. In some cases it may be found convenient to widen the incision temporarily by means of caponizing spreaders. Following this, a thread loop is put around the two ribs to draw them closer together, to prevent "bloating." The incision is then sewed up in the ordinary way.

The experiment was designed to compare the fertilizing power of the treated and control spermatozoa according to the two methods of insemination. There were four groups of birds. Group (*a*) received the radiated semen intraperitoneally; group (*b*), control semen (untreated) intraperitoneally; (*c*) radiated semen, via the usual (vaginal) route; (*d*) same as (*c*) except that the semen was untreated. Groups (*a*) and (*b*) received 0.5 cc. of the semen, while groups (*c*) and (*d*) received the usual 0.1 cc. The treated spermatozoa were subjected to two levels of irradiation: 5,658 r and 9,340 r.

The average operation time was 15 minutes. The birds were then placed in individual cages, where a close check was kept over their condition and egg-laying. Altogether, twenty-nine birds were used in the two tests. Tables 2 and 3 summarize the results obtained. The tables are presented in the form of egg-laying records not only to indicate the effect of different treatments on the fertilizing capacity of the spermatozoa but also to show the rate of egg-laying immediately preceding and following the operation. There was some evidence that the operation interfered with the normal ovulation rate of the operated birds. Only one bird, however, died sufficiently soon after the operation to ascribe the death to the operative trauma.

TABLE 1

EFFECT OF X-RAYS ON COCK SPERMATOZOA: SUMMARY OF THE DATA

CRITERION	TREATMENT	DOSE IN ROENTGEN UNITS								
		231	462	924	1,848	2,772	3,696	4,620	5,544	6,488
No. of eggs set.	X-rayed	50	86	94	118	126	88	194	134	84
	Control	36	76	90	124	136	84	182	138	88
Percentage of eggs fertile...	X-rayed	76.0	67.4	68.1	64.4	27.0	29.6	21.7	11.9	0
	Control	100.0	81.6	91.1	69.4	88.2	85.7	95.6	87.0	0
Dead embryos as percentage of fertiles....	X-rayed	21.4	28.0	50.0	63.2	76.5	78.0	83.5	100.0	....
	Control	25.0	4.0	26.8	23.3	20.0	13.9	17.2	28.3	49.0
Secondary sex ratio........	X-rayed	63.6	61.5	66.7	64.7	66.7	50.0	42.9	....	....
	Control	50.0	55.3	53.8	52.5	53.0	53.0	49.0	....	....

Concerning the more interesting point of egg fertility in the experimental (radiated) groups, there is obviously no difference in this respect between the two methods of insemination. In both the fertility was nil, thereby showing that the method of introducing spermatozoa is not a factor responsible for the reduced proportion of fertile eggs in the experimental group. This point will receive further consideration later.

Another possible way in which radiation could affect the fertilizing powers of cock spermatozoa is the induction of changes in the seminal fluid detrimental to the spermatozoa. This possibility de-

TABLE 2

VAGINAL VERSUS INTRAPERITONEAL ROUTE OF INSEMINATION: FIRST TEST

GROUP*	HEN NO.	DAYS BEFORE AND AFTER INSEMINATION†													
		6	5	4	3	2	1	0	1	2	3	4	5	6	7
a	Z928		1			1	1	1						I	
a	Z1124								1	I		I			I
a	Z1640		1	1	1	1	1	1		I					
a	AA959	1	1	1	1	1	1	1	1	I					
a	AA956		1	1			1	1		I		I	I		I
b	Z990		1	1		1				I		F		F	
b	Z1062		1	1	1	1	1	1	1				F		F
c	Z76		1	1	1			1		I	I		I		I
c	Z1061				1	1					I			I	
d	Z317		1		1		1				F	F		F	
d	Z397		1	1			1			F		I		I	F

* Dose: 9,340 r.

† The figure 1 indicates egg laid on that day. Refers to eggs laid prior to insemination. "I" means the egg was infertile; "F" means the egg was fertile.

TABLE 3

VAGINAL VERSUS INTRAPERITONEAL ROUTE OF INSEMINATION: SECOND TEST

GROUP*	HEN NO.	DAYS BEFORE AND AFTER INSEMINATION†													
		6	5	4	3	2	1	0	1	2	3	4	5	6	7
a	Y663		1		1		1		I	I			I	I	
a	Z317		1	1											
a	Z518	1	1	1			1	1	I		I		I	I	
a	Z1061		1			1									
a	AA988		1	1	1	1		1	I						
a	AA2063		1	1		1	1								
b	Z76	1		1			1		I		F		F		I
b	Z484		1	1	1	1		1							
b	Z507		1				1		D‡						
b	Z928	1		1	1	1	1		I	F					
b	Z1127		1	1		1	1								
b	Z1166		1		1	1		1							
c	Z990		1		1		1	1		I					
c	Z1124	1		1		1	1	1		I	I		I	I	I
c	Z1640	1		1	1		1	1	I		I	I	I	I	I
d	Z956	1	1	1	1		1	1	I	F		F	I	F	F
d	Z959	1		1	1	1	1			F		F	I	I	I
d	Z1062	1	1	1	1	1		1	I			F	F	I	F

* Dose: 5,658 r.

† Same symbols as in Table 2.

‡ Died.

serves some attention on the basis of the known capacity of Roentgen rays to produce chemical changes in organic media. In this respect the seminal fluid can be regarded as the medium for the spermatozoa.

Claus (1933) has shown that soft X-ray radiation produces changes in the solutions of heavy salts which normally can support the growth of *Bacillus coli*, making these solutions unsuitable as media. A similar observation was made on a number of different bacterial media by Blank and Kersten (1935). A parallel series of studies was made on protozoan media by Pugsley, Oddie, and Eddy (1935) and by Taylor, Thomas, and Brown (1933).

The study most pertinent in this connection is that of Evans, Slaughter, Little, and Failla (1941), in which it was found that the effect of X-rays on the fertilizing capacity of *Arbacia* sperm may be mediated indirectly, through a harmful substance formed in the medium (water) following irradiation. Kagami (1938) reported that the pretreatment of sea water with X-rays prior to introduction of sea-urchin eggs accelerates the subsequent mitosis of the eggs.

The centrifugation technique is the only practical way in which the effect of radiation on either of the two major components of the cock semen can be studied independently. Munro's work (1938) and later tests at the Poultry Division have shown that almost complete separation of the two components can be accomplished by a 20- to 30-minute centrifugation at 2,000–2,500 r.p.m. The microscopic examination of the seminal fluid following such treatment shows it to be almost completely azoospermic.

Prior to centrifugation, each specimen of the semen was divided into two unequal parts. The smaller portion served as a negative control, receiving no treatment whatsoever. The larger portion was again divided, this time into two equal lots. One-half was irradiated with 2,829 r. Both halves were then centrifuged, and the fluid portions of the two specimens were mutually exchanged. All three samples—i.e., the negative control and the two centrifuged portions—were then inseminated in the usual manner (via the vaginal-uterus route) into a number of White Leghorn hens. A record of fertility was kept of all the eggs laid by the hens during the usual 10-day period. Two independent tests were carried out, involving 47 hens and 296 eggs. Pooled results are summarized in Table 4.

Results indicate that in these tests the effect of X-rays was applied directly to the gametes. Irradiation of the seminal fluid alone failed to alter the fertilizing powers of the untreated spermatozoa. A small drop in the proportion of fertile eggs secured in the group in which the untreated spermatozoa were added to the radiated fluid undoubtedly resulted from the postirradiation treatment of the specimens. Tests involving nonradiated semen showed that the process of centrifugation in itself somewhat lowers the fertilizing power of the semen.

SPERMATOZOAN VIABILITY IN VIVO

Evidence secured in an indirect way and presented in the foregoing sections seemed to indicate that the progressive drop in the fertilizing capacity of the irradiated spermatozoa cannot be accounted for on the basis of reduced viability of such spermatozoa. The evidence was supplied by the results obtained from the intraperitoneal inseminations of normal hens with the X-ray–treated gametes. Below will be presented further material bearing on the subject of viability of the radiated spermatozoa in the reproductive tract of the hen.

Let it be assumed for a moment that the X-ray treatment lowers the survival index of the spermatozoa. Then it should follow that such effect will become progressively more apparent the longer the semen remains in the oviduct. Fertility of the ova is a good indication of the functional activity of the artificially introduced spermatozoa. Thus, if the postulate is correct, relatively speaking, fewer fertile eggs will be obtained in the experimental group in the latter part of the 10-day period. To be valid, the drop would have to exceed reduction in fertility normally expected under similar circumstances in control matings.

TABLE 4

EFFECT OF X-RAYS ON FLUID PORTION OF COCK SEMEN

Group*	No. of Birds Inseminated	No. of Eggs Laid	Percentage of Eggs Fertile
Control	11	73	86.3
Untreated fluid with radiated spermatozoa	18	109	28.4
Radiated fluid with untreated spermatozoa	18	114	82.5

* Dose: 2,829 r.

To test this point, the usual 10-day period of observation was arbitrarily divided into two equal parts. This was done to compare the proportion of fertile eggs appearing in each half. Both the control and the experimental groups were examined from this standpoint. The result of this comparison is summarized in Figure 2. The data seem to indicate that, in so far as the material on hand is concerned, the X-ray treatment of spermatozoa had no effect on their subsequent functional life-span, if the latter is limited to 10 days following insemination. Although there was a considerable drop in the proportion of fertile eggs laid in the second half of the observation period, this trend was apparent in both groups of eggs, irrespective of the treatment.

Additional corroboration of this observation can be obtained from the material concerned with prenatal mortality. As will be shown shortly, radiation of cock spermatozoa results in a considerable rise in the lethality of zygotes. Therefore, if the radiation treatment has no effect on the spermatozoan functional life-span, distribution of lethal embryos should be even among the eggs laid during the observation period. The radiated spermatozoa would be expected to continue "siring" the same proportion of lethal embryos during the entire length of this period.

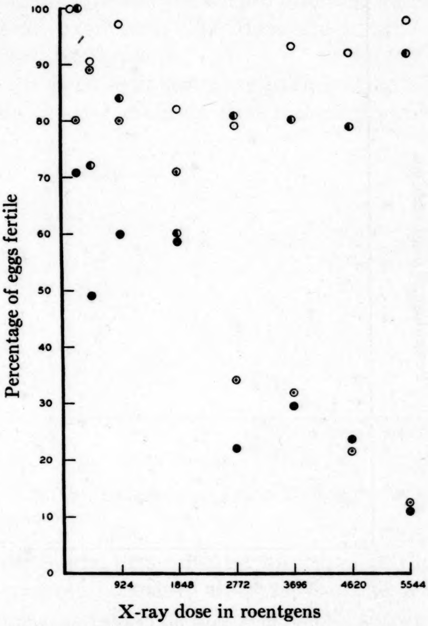

FIG. 2.—Effect of X-rays on fertility according to time incidence of laying after treatment. ○ = control, 1–5 days; ◐ = control, 6–10 days; ⊙ = experimental, 1–5 days; ● = experimental, 6–10 days.

Data summarized in Figure 3 indicate

that there is no undue difference in the distribution of lethal zygotes. Thus, an egg laid 2 days after insemination is just as likely to contain a lethal embryo as an egg laid on day 9 or 10. Because it is now known that fertilization in the hen occurs soon after ovulation, the radiated spermatozoa would have to remain functionally active during the 10 days in question, to produce this result.

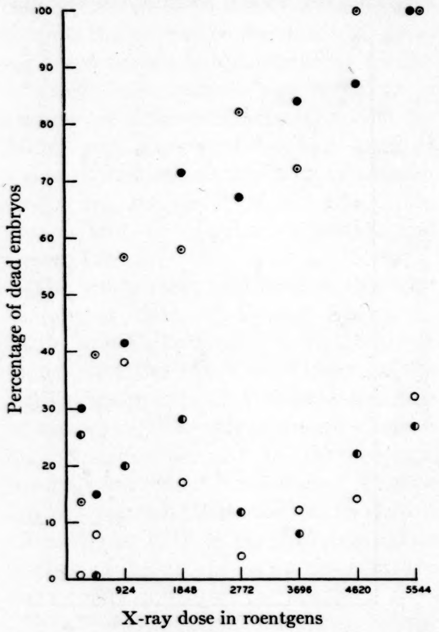

FIG. 3.—Effect of X-rays on embryo mortality according to time incidence of laying after treatment. ○ = control, 1–5 days; ◐ = control, 6–10 days; ⊙ = experimental, 1–5 days; ● = experimental, 6–10 days.

A somewhat different picture, however, is obtained if the period of observation is extended beyond 10 days, to include all the fertile eggs laid by the individual hen following a single insemination. To secure the necessary data, the eggs were saved from each hen for as long as the daily settings of them indicated persistence of fertility. In some cases this meant saving the eggs for 3 weeks or more after insemination. As a general rule, at least three successive eggs were saved following the appearance of the last fertile egg.

In the present case, comparisons were made on the basis of the number of days which elapse between insemination and the laying of the last fertile eggs. The material is presented in Figure 4. It shows that there was a distinct falling-off in the length of the functional life-span of the irradiated spermatozoa as the intensity of radiation increased. This effect was persistent throughout the dose range used in this investigation. Moreover, there is a suggestion that the dose of 1,840 r is critical in this regard.

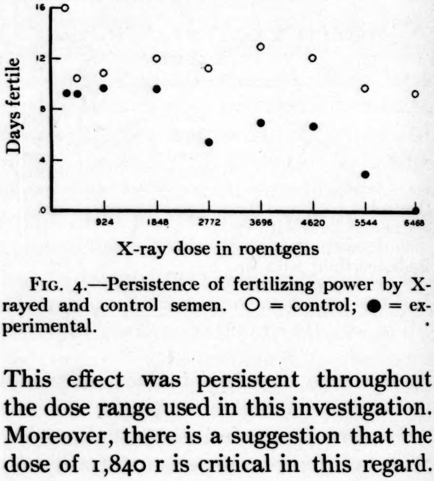

FIG. 4.—Persistence of fertilizing power by X-rayed and control semen. ○ = control; ● = experimental.

SEX RATIO

Some interesting data have been obtained in the course of this series of studies bearing on the subject of secondary sex ratio. The material is summarized graphically in Figure 5. On the whole, the distribution of points shows a definite modification in the secondary sex ratio of zygotes fathered by the X-ray radiated spermatozoa. The ratio, on the average, was 62 per cent—some 12 per cent deviation from the corresponding value in the controls. This difference, when tested statistically, was found to have odds of 30:1 on significance. It should be pointed out that the extreme right wing of the graph in the treated group was based on a small number of individuals because of low fertility and "hatchability" at higher doses. This fact, in all probability, accounts for the change in the relationship of the two curves.

EFFECT OF THE X-RAYING OF SPERMATOZOA ON THE PRENATAL DEVELOPMENT OF SUBSEQUENT ZYGOTES

INTRODUCTION

There is much evidence that short-wave radiation of either one of the gametes has adverse effect on zygotic viability. Bardeen (1907) and McGregor (1908), working with toads and frogs, respectively, found that the ova fertilized by radiated spermatozoa seldom produced viable tadpoles. These observations were soon corroborated and extended by the Hertwig group, by Packard (1914, 1918), and, later, by Little and Bagg (1923), Mavor and De Forest (1924), Muller (1928), Hanson (1928), Barth (1929), Hanson and Heys (1932), Henshaw (1932), Strandskov (1932), Mori, Miwa, and Yamashita (1939), Rugh (1939), Rugh and Exner (1940), Henson (1942), and others. Snell (1941) reported smaller litters from mice mated to males previously subjected to Roentgen radiation. A parallel observation was made earlier on *Drosophila melanogaster* by Sonnenblick (1938). Further evidence on the matter is supplied in a recent study by Henson (1942).

As far as the author is aware, no similar study has been undertaken on the domestic chicken. Because the question is of some importance from the standpoint of the broader problem with which this report is dealing, data were collected bearing on the topic.

QUANTITATIVE STUDY OF EMBRYONIC LETHALITY

The procedure employed was as follows: Yolks with dead or obviously abnormal embryos were carefully examined *in situ* for any irregularities in the pattern of extra-embryonic vascularization, orientation of the embryo, or the presence of other gross abnormalities. Altogether, more than two hundred eggs were examined. The data are summarized in Figure 6.

It is evident from the examination of the scatter diagram that more embryos die in the experimental lots than in the controls. There is an obvious trend for the mortality rate to rise with the increased intensity of irradiation. In this connection it is interesting to note that at the level of 5,544 r, the critical point for the fertilizing power of cock spermatozoa, no embryos had survived to hatching time. The increased rate of mortality among experimental zygotes, however, is not entirely due to treatment. There is a fair amount of parallelism between the two groups, beginning with the dose level of 2,772 r. Obviously, the factor or factors responsible for embryonic mortality among control chicks are still operative in the experimental lot. The X-ray-induced injurious effects were

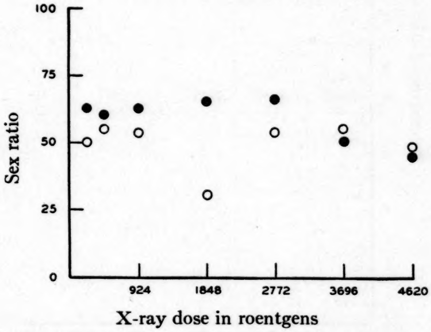

FIG. 5.—Secondary sex ratio. ○ = control; ● = experimental.

merely superimposed on the already present condition.

The data are re-examined in greater detail in Figure 7, this time the focus of attention being concentrated on the time incidence of deaths. For this purpose the incubation period was considered to be made up of two almost equal periods (10 and 11 days). The occurrence of mortality was then compared within each treatment group with reference to these divisions. For the sake of clarity the distribution points of only the first (10-day) period was considered in Figure 7. Because all values are expressed as fractions of unity, the distribution of corresponding points in both periods is obviously complementary.

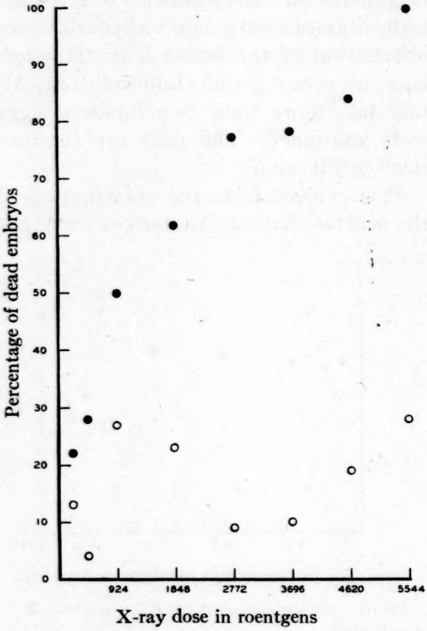

FIG. 6.—Effect of X-rays on embryonic mortality. ○ = control; ● = experimental.

Figure 7 shows that the bulk of the prenatal embryonic deaths in the experimental lots occurs sometime during the first 10 days of incubation. The detailed data show that by far the largest majority of deaths occurs in the first 4 or 5 days of embryonic life. Embryos which survive this critical period have a good chance of living to and beyond hatching time. In the control lots, on the other hand, the rate of mortality, on the whole, was evenly spread throughout.

The fact that fewer embryos die in the second 10-day period of the embryonic life suggests that the embryos which remain alive hatch into normal chicks. Lethal or semilethal zygotes are weeded out early. The possibility exists, of course, that some embryos survive, carrying latent (nonlethal) mutations which can be brought out only by proper breeding tests. No information as yet is available on this point. The evidence on

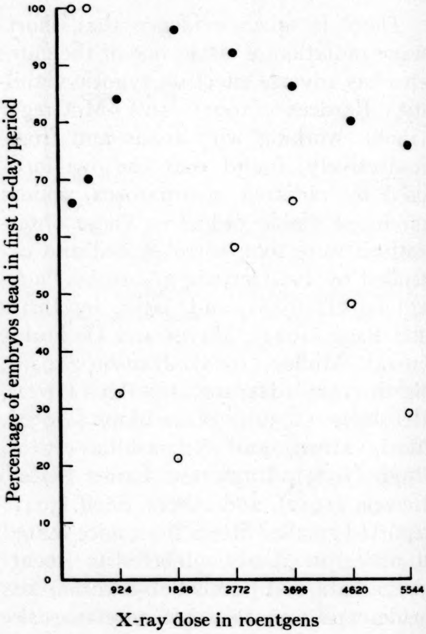

FIG. 7.—Time incidence of embryonic mortality. ○ = control; ● = experimental.

hand shows, however, that the chicks which do hatch compare favorably with their control siblings.

Some data were obtained on the growth rate of the comparable lots of experimental and control chicks. The material is summarized in Table 5. There were no striking differences in the growth rate between the two treatment groups.

The general plan of these tests, with slight variations, was as follows: Pooled semen from a number of Barred Rock males was irradiated with a known quantity of X-rays. Only half of the total volume of the available semen at any one time was so treated. The other half received no irradiation and served to inseminate a control group of hens. Again, White Leghorn pullets provided the eggs.

TABLE 5

RELATIVE GROWTH RATES OF CHICKS FROM EXPERIMENTAL AND CONTROL GROUPS*

Test No.	Treatment	Eggs Set	Percentage Fertile	Percentage of Dead Embryos	Chicks Hatched	Put in Brooder	Body Weight in Grams			
							0 Weeks	4 Weeks	6 Weeks	9 Weeks
1......	Radiated	67	65.7	15.9	37	37	40.1	230.1	....	....
	Control	48	79.2	2.6	37	37	39.8	240.4	....	....
2......	Radiated	97	57.7	46.4	30	23	40.0	232.4 (22)†	....	....
	Control	50	80.0	10.0	36	24	40.9	250.4 (22)	....	....
3......	Radiated	169	57.4	22.1	53	30	39.3	181.7 (17)	378.8 (15)	642.3 (14)
	Control	125	66.4	8.3	73	26	39.0	175.9 (21)	358.9 (16)	635.8 (13)

* Dose: 1,414 r.

† Number of chicks on which body weight was based. Reduced numbers are due to mortality and other reasons.

QUALITATIVE STUDY OF EMBRYONIC LETHALITY

The second portion of this section of the report deals with the optically detectable modifications in the development of fertilized ova. As already pointed out, the bulk of deaths among experimental embryos occurs in the first few days of prenatal ontogenesis. Therefore, it seemed reasonable to assume that any deviation in the developmental pattern induced by X-ray radiation of the male gamete will be largely confined to the early portion of that development. A series of five independent tests was planned to study the subject in greater detail.

Altogether, 154 fertile eggs were systematically examined, of which 70 were studied histologically.

The dose range, to which the spermatozoa were subjected, varied from 78 r to 4,715 r. All eggs were set together in the same incubator, irrespective of their classification. As in previous tests, eggs were collected for 10 days following insemination. As a rule, all eggs were set daily and then withdrawn for examination and study at definite intervals. The first group so withdrawn was incubated for 24 hours. In some tests the eggs were allowed to accumulate for 10 days before setting, in which case the samples withdrawn were selected to be representative of all the eggs laid on different days following insemination. In the first four experiments of this series, five groups in all were withdrawn at 24-hour intervals. Thus, the last blastoderms examined were incubated for 120 hours. In the fifth test it was found more convenient to adopt a less rigid schedule for withdrawals.

As soon as the eggs were removed from the incubator, they were examined for fertility while still in shell. The shell of the fertile eggs was removed around the large end, to expose the blastoderm, which was then examined for abnormal development. Abnormal blastoderms were prepared for histological examination.

Before proceeding with the description of such abnormalities, a brief reference will be made to the normal type of early development. Experience at this laboratory has shown that, macroscopically, the fertile ova can be identified before incubation (Kosin, 1944). The blastoderm measures from 2 to 3 mm. in diameter. In most cases, on the perimeter of the centrally located germinal disk there is a thin, diffused polar ring. When a histological preparation is made at this stage, embryonic cells are already laying ground for future germ-layer differentiation. Bulk staining technique reveals the cells scattered in clusters over the surface of the blastoderm. After 6 hours of incubation the blastoderm has already expanded to measure 5–6 mm. across. The process of layer differentiation continues. The centrally located cells, immediately under the vitelline membrane, grow smaller in size, while the marginal cells thin out to a single layer and continue to expand peripherally at the expense of the periblast. A one-cell-thick layer stretches over the segmentation cavity. By the time the eggs have been 10 hours in the incubator, the blastoderm measures 6–8 mm. in diameter. This dimension increases to 10–14 mm. after 24 hours of incubation. Blood islands are visible in the caudal portion of area opaca. By the forty-eighth hour of incubation the blastoderm has expanded to measure about 35 mm. in diameter, with the area vasculosa that incloses the central portion of the blastoderm measuring about 11 mm. Blood circulation has already been established, and the major extraembryonic blood vessels are in evidence. Over three-quarters of the yolk surface is covered by the blastoderm after 72 hours of incubation, with the area vasculosa measuring approximately 25–30 mm. across. The embryo is now about 10 mm. in length.

Criteria on the basis of which classification was made concerning the state of normality of the developing embryos were of two kinds: one was the over-all size of the blastoderm; the other was the type and degree of differentiation. Both of these were considered, of course, in conjunction with the recorded incubator age.

The size of the blastoderm, i.e., its diameter, is a useful criterion in spotting abnormalities, when it is considered together with other evidence. Thus, an egg incubated for 48 hours and possessing a blastoderm measuring only 10 mm. in diameter very likely represents the case of an early death. If, however, in addition to the small size of the blastoderm, there are signs of blood islands and evidence pointing to the presence of definite area pellucida, abnormal development can be suspected. The same line of reasoning would apply to classifying a 48-hour blastoderm, which measures 30 mm. in diameter, without any signs of differentiation, as "abnormal." Blastoderms which presented a gross picture of "normality" were passed over without any further examination. It will be recalled, however, that 70 blastoderms, of the 154 examined in this series, were sectioned and studied histologically. Of these, 40 were found to be definitely abnormal, all but 2 of these being from the experimental ova.

In most cases it was found impractical to section whole blastoderms, largely because of technical difficulties. Therefore, representative samples, taken from various areas of the blastoderm, were sectioned and examined histologically. In a number of cases serial preparations were made, while in others only a certain portion of the sections, taken in a definite order, were mounted. The type of abnormalities encountered are listed in Table 6.

The total absence of the truly embryonic tissues accounts for the largest single class of abnormalities observed in this study. On examination, such embryoless blastoderms usually show definite sinus terminalis and blood islands, which serve as landmarks in determining the age of the blastoderm. Usually there is also a more or less clearly marked area pellucida. The more careful examination of this area on histological sections reveals a complete absence of true embryonic tissue. On the other hand, the presence of blood islands indicates a considerable degree of differentiation. The ectoderm is represented by a thin layer of cells overlying a network of yolk-laden, presumably entodermal cells. This arrangement resembles the type of cellular differentiation found in the area peripheral to the locus of normal embryonic growth. Sections of samples taken from area vitellina interna and externa show no deviation from normality.

The second largest group of abnormalities includes examples of the almost complete absence of differentiation either in the embryonic region or in the area vasculosa. In these cases, although the over-all size of the blastoderm compares favorably with the corresponding incubator age of normal blastoderms, there is little organizational differentiation. There is a complete lack of erythropoietic activity. This suggests absence of mesodermal differentiation, if blood islands are taken to be of mesodermal origin (Lillie, 1919, p. 88). A similar situation with respect to mesoderm was observed in three embryonic vesicles in the rats mated to males treated with Roentgen radiation (Henson, 1942).

TABLE 6

FREQUENCY OF FOUR GENERAL TYPES OF ABNORMALITIES IN 40 EMBRYOS

Type of Abnormality	Frequency
Size of blastoderm is normal, but no differentiation into germ layers	13
Size of blastoderm is normal; blood islands present; no truly embryonic tissue present	18
Size of blastoderm is normal; embryos lack notochord	4
Deficient extra-embryonic blood vascular system or complete absence of erythroblasts	5

Moreover, Grodzinski (1933, 1934) produced total suppression of mesoderm in chick blastoderm by exposing eggs to low temperature prior to incubation. Such blastoderms lacked embryonic differentiation, i.e., no primitive streak was formed.

The remaining two groups of abnormalities were of relatively rare occurrence. The observed absence of notochord in four embryos was associated with the failure of the neural plate to roll into a tube. This situation resembles somewhat the spina bifida condition in amphibians.

Absence of embryonic cells and the incomplete development of large extra-embryonic blood vessels was a phenomenon observed in five embryos. Although such embryos appeared normal, the embryonic blood vessels lacked erythroblasts, probably because of the failure of the extra-embryonic vessels to establish connection with the omphalomesenteric veins. In two cases contractions were observed in bloodless hearts.

All of the above cases of abnormal development were found among eggs belonging to the experimental group. As already mentioned, there were two instances of abnormal blastoderms in the control group. On gross examination both specimens showed small blastoderms, measuring but 15 and 16 mm. after 48 hours of incubation. Area vasculosa, about 5 mm. across, contained small embryos 2 and 3 mm. in length.

The type of abnormal development found in the experimental group appeared to bear no relationship to the dosage.

EFFECT OF X-RAYS ON THE SPERMATOZOA IN VITRO

OBSERVATIONS ON SPERMATOZOAN MOTILITY

Although motility of spermatozoa is not an infallible index of their fertilizing capacity, it is the most obvious criterion of it. Obviously, immotile cells cannot fulfil their function. Degree of fertility in natural matings appears to be predicated upon motility of male germ cells (Hartman, 1939).

A number of investigators have studied the problem of spermatozoan motility as it is affected by short-wave radiation. Bardeen (1907) found that Roentgen radiation lowers motility of frog spermatozoa. Hooker (1925) was unable to secure consistent results on motility of rat spermatozoa irradiated with X-rays. More recently Fuke (1930) observed that X-rays exert no influence on the motility of rabbit spermatozoa. A similar conclusion was reached by Asdell and Warren (1931), who subjected rabbit semen to "huge amounts" of X-rays (from 3.5 to 11 erythema doses). Still more recently, Rugh (1939) did not notice any appreciable changes in the motility of frog spermatozoa treated with X-ray doses as high as 50,000 r.

In the present investigation, data on motility were gathered along with the previously reported study on the effect of X-rays on the fertilizing power of cock semen. Small portions of semen were set aside from the irradiated and untreated specimens. Each such portion was then divided into two equal parts, which were transferred into 4-cc. test tubes. One tube was left standing at room temperature, while the other was placed in a refrigerator in which the temperature was maintained at 5° C. Observations on motility were made at 0, 18, 44, and 60 hours. In a few exceptional cases spermatozoa remained motile for 180 hours. Generally speaking, however, 60 hours was the end point for all observations.

Motility was graded according to four classes (+, ++, +++, ++++) suggested by C. R. Moore (1928). Although there was considerable variation in motility between individual samples, each dose was repeated a sufficient number of times to furnish ample data for statistical analysis. For this purpose, numerical values were assigned to each motility group (thus, ++++ became "4"; +++, "3"; and so on). The plan of the experiment lent the material to a statistical test by "Student's" method for paired observations. Motility indices were compared in the usual manner. Results of these comparisons are summarized in Table 7.

As was anticipated, room temperature of 22° C. proved to be more detrimental to spermatozoan motility than 5° C., irrespective of the treatment. Only a few samples of those observed at 60 hours at room temperature showed any motility. At 5°, however, a large number of samples contained motile spermatozoa 84 hours after ejaculation.

With reference to Table 7, it should be pointed out that it is based on the motility values obtained from samples used in the dose range beyond 8,300 r. There was no appreciable difference between the motility of spermatozoa subjected to doses ranging from 231 r to 8,300 r and that of their control counterparts.

TABLE 7

COMPARISON OF SPERMATOZOAN MOTILITY ACCORDING TO TREATMENT, HOLDING TEMPERATURE, AND LENGTH OF STORAGE*

No. of Hours Held	Temperature	No. of Pairs Observed	Mean Motility Difference in Favor of Controls	*Z*-Value (Odds in Brackets)
0........	....	22	0.18	0.36 (15.4:1)
18........	Room	26	0.65	0.87 (5,000:1)
	5° C.	24	0.33	0.42 (34:1)
44........	Room	14	0.29	0.62 (44.5:1)
	5° C.	23	0.26	0.48 (56.0:1)
60........	Room	....	....	....
	5° C.	17	0.47	0.50 (30.8:1)
84........	Room	....	....	....
	5° C.	11	0.55	0.52 (14:1)

* Dose range: 8,300–36,900 r.

However, when observations on motility were extended beyond 8,300 r and up to 36,900 r (Table 7), there appeared a distinct decline in the motility of the radiated cells. Only the 84-hour sample failed to show statistically significant difference. This was probably due to the small number of observations, although, actually, the mean difference in the motility indices of the two treatment groups was high.

OBSERVATIONS ON THE MORPHOLOGY OF SPERMATOZOA

Parallel with these studies, information was gathered on the comparative morphology of the irradiated and control spermatozoa. Smears of semen were made according to a method described by Munro (1936). Light green dye brilliantly stains the acrosome, the neck, and the flagellum, while hematoxylin stains the nucleus.

Normally, the stained preparations of fresh cock semen show the spermatozoa to be scattered singly and in groups over the entire field of observation. The head piece is straight; the long flagellum also shows almost no contortions. There is, naturally, a wide range of variation in the appearance of single cells. The actively moving cells are "fixed" in lifelike positions of arrested motion.

When stained smears are made of semen held for 12 or more hours at the temperature of the refrigerator, certain modifications begin to appear in the over-all picture of the preparation. These changes are limited to individual cells. The head portion shows an increased tendency to curl up into circles. A great deal of contortion is exhibited by the flagellum. With the increased length of the storage period the proportion of cytolyzed cells is raised. This condition appears to be merely a continuation of the general process of cellular destruction. Cytolyzed cells still take the light green stain, though to a lesser extent than the living cells. However, they rarely stain with hematoxylin, so that the normally basophilic nuclear portion now almost completely lacks color.

With X-ray doses up to 38,000 r—i.e., well beyond the biotic range—no undue morphological changes were noticed in the appearance of the cells when preparations were made within a few hours after ejaculation and irradiation. However, commencing with the dose of 13,000 r, there was observed a marked tendency on the part of the radiated cells to form tight circles in the preparations made 18 hours following ejaculations. Although, as already pointed out, these types occur in normal specimens on storing, their frequency is distinctly stepped up with the rising intensity of radiation.

In addition to the aforementioned observations, a number of smears were made of the semen receiving 76,000 r, 114,000 r, and 152,000 r. In these groups the required energy was provided in single applications by a 200-kv. unit. Here the characteristic changes in the appearance of the individual cells were noted as early as 3 hours following the treatment. All smears showed large masses of clumped spermatozoa exhibiting extreme contortion. The fertilizing capacity of these specimens was nil.

EFFECT OF RADIATION ON pH OF SEMEN

The irradiation treatment within the biotic range (as established in this investigation) had no appreciable effect on the hydrogen-ion concentration of the semen. The pH of both the experimental and control lots was found to vary between 6.10 and 7.20. This range is somewhat wider than that reported for this type of material by Zagami (1937). He found the pH of the cock semen to average between 7.02 and 7.18. All measurements were taken with a Beckman pH meter. The pH tended to shift to the alkaline side when measurements were compared at 0, 24, and 48 hours following ejaculation. This corroborates the earlier observation of Zagami (1937). The extent of the shift was similar in both treatment groups.

EFFECT OF X-RAYS ON CERTAIN METABOLIC PROCESSES OF COCK SPERMATOZOA

INTRODUCTION

It has alrady been shown that Roentgen radiation lowers and, above a certain dose, completely blocks the fertilizing capacity of cock spermatozoa. At the same time, motility does not seem to be as easily influenced by this type of radiation. In fact, as was shown above, within the physiological or biotic dose range, i.e., the range of doses within which spermatozoa retain some of their functional capacity, motility is not affected at all. This behavior pattern is correlated with the morphological appearance of the spermatozoa. In view of these largely negative results, it was felt that perhaps the explanation for the extreme sensitiveness of the cock spermatozoa to X-ray radiation may lie in the more fundamental processes pertaining to cellular physiology. The importance of enzyme action in the biology of the gametes is receiving an increasing amount of attention because such reactions are perhaps at the base of their activity.

The type and the number of enzymatic reactions concerned with the behavior and function of spermatozoa is still, largely, an unexplored field. However, spermatozoa are known to carry on, in common with other living cells, certain fundamental chemical reactions. Some of these reactions were studied in the present series.

No generalization can be made with regard to the effect of short-wave radiation on cellular metabolism. A number of investigators—Adler (1930), Bancroft and Kinsey (1937), and Bodine, Carlson, and Ray (1940)—have reported an adverse effect of X-ray radiation on the metabolism of tissues or of the isolated groups of cells. In all these studies it was found that the metabolic rate of the radiated material was definitely reduced by the treatment.

More specifically, this type of shortwave radiation was found to lower or completely block the activity of certain enzymes. Thus, according to Clark and Northrop (1925), soft X-rays inactivate trypsin; Albers (1940) concluded that these rays inhibit the action of serum phosphatase. Similar findings were made with crystalline carboxypeptidase and polyphenoloxidase (Dale, 1940); succinate dehydrogenase (Havard, 1935); tyrosine (Ray, 1938); urease and catalase (Tytell and Kersten, 1941).

In contrast to these results, evidence can be mustered to show that X-rays exert no measurable influence on enzymes, particularly those concerned with respiration and glycolysis. Roth and Morton (1923) stated that any changes in the enzymatic activity of pepsin solutions subjected by them to X-ray radiation were within experimental error. Wels (1924) was unable to demonstrate any modification in the respiratory rate of irradiated muscle and red blood cells. Lactate and glucose citrate dehydrogenases are apparently immune to Roentgen rays of 20,000-r intensity (Havard, 1935). The latter worker concluded that the biological effects of X-rays are not caused by any primary action upon the enzymes of respiration. He was corroborated by Reis and Kluge (1936), Scott (1937), and Boell, Ray, and Bodine (1937). Goldfeder and Fershing (1938) failed to alter the oxygen consumption of mouse-liver slices by exposing them to heavy X-ray doses.

The present investigation limits itself to the study of certain phases of respiration and anaerobic glycolysis.

GENERAL METHODS

The over-all procedure pertaining to semen collection, radiation, etc., did not differ through the series. Consequently, only a brief description of the method will be outlined here, to avoid repetition when dealing with individual tests.

The semen was obtained from a flock of yearling Brown Leghorn males kept at the Whitman Laboratory of the University of Chicago. As soon as the ejaculate was secured, it was strained through medicinal gauze and then cooled. Generally speaking, the semen was obtained early in the afternoon and irradiated within 45 minutes. The X-ray machine that supplied the energy was a 200-kv. animal-therapy unit operated at 4 ma. A filter, consisting of 3-mm. aluminum plate, was used in all radiations.

Before irradiation the ejaculate was divided into two equal portions; one served as control, the other was subjected to the X-ray treatment. The latter was placed in a shallow celluloid vessel, which was, in turn, placed in an open Petri dish. Cracked ice was packed around the celluloid vessel to keep the sample reasonably cool during radiation. The Petri dish was brought to within 9 cm. of the source of the energy. At this distance the output of the tube was approximately 2,000 r per minute. The standard dose adopted in this series was 10,000 r, or nearly double the critical (maximum biotic) dose (see Table 1). Upon irradiation the samples were placed in a cold-storage room, where they remained until manometric determinations could be made. This usually meant an interval of from 2 to 6 hours between the ejaculation and the first manometric reading. The mode was approximately 3 hours.

The Warburg type of manometric equipment was used, all tests being run in triplicates—i.e., three manometers were assigned to the treated semen and an equal number to the control. The seventh manometer served as a thermobarometer. Temperature of the water bath was kept at 37.5° C. Although this temperature was below that of the avian body, the fact that both the treated and the control lots were subjected to the same temperature treatment made the results comparable within each experiment.

Manometric readings were recorded

every 10 minutes for 60 minutes after a 10-minute equilibration period. Dry-matter content of the semen was the basis for calculating both the relative and the absolute rates of reaction.

EFFECT OF X-RAYS ON QO_2 OF COCK SEMEN

Respiration determinations were made in specially constructed small vessels, the volume of which ranged from 6.816 to 7.723 cc. The semen was diluted with a buffered Ringer's solution (pH 7.4), in the ratio of three parts of the diluent to one part of semen. During preliminary trials it was found that it was not necessary to provide glucose to the substrate, although Winberg (1939) reported that the chicken spermatozoa can utilize a number of exogenous monosaccharides, including glucose. The rate of reaction was considered satisfactory even in the absence of added glucose.

TABLE 8

QO_2 OF IRRADIATED AND CONTROL SEMEN*

TEST NO.	IRRADIATED SERIES†				CONTROL SERIES			
	Individual Vessels			Mean	Individual Vessels			Mean
1	5.74	5.25	6.06	5.68	4.11	4.36	4.14	4.20
2	2.62	2.79	....	2.71	3.41	2.77	....	3.09
3	2.82	2.50	2.71	2.68	2.47	2.61	2.20	2.43
4	1.64	1.83	1.79	1.75	2.11	2.28	2.49	2.29
5	2.17	2.23	2.23	2.21	2.24	2.53	2.49	2.42
6	1.98	1.80	1.77	1.85	3.15	2.91	2.98	3.01
7	1.94	1.79	1.97	1.90	2.01	2.01	1.87	1.96
8	1.63	1.66	1.33	1.54	1.64	1.49	1.92	1.68
9	3.97	4.13	3.93	4.01	4.73	5.12	5.23	5.03

* Only Ringer's solution added.

† Dose: 10,000 r.

Each vessel received 1 cc. of the semen-Ringer suspension. In addition, 0.15 cc. of 15 per cent KOH were placed in the center cup of each vessel to absorb CO_2 given off during the reaction.

Table 8 summarizes the results of QO_2 determinations. The figures represent the cubic millimeters of O_2 consumed in 60 minutes per milligram of dry weight. Nine experiments were carried, all but one in triplicate.

Examination of Table 8 reveals extreme variability in the rate of reaction when comparisons are made between independent experiments. However, there is a close degree of agreement in the performance of samples of the same experiment. This pattern of reaction was expected. The existence of big differences in the rate of O_2 consumption of semen obtained on different days can be largely, if not wholly, accounted for by the variability in the relative contributions of semen from individual males. By and large, the consistency of ejaculate from an individual cock does not vary a great deal from day to day. However, the volume of such ejaculates varies; and, since all specimens were pooled, naturally the over-all consistency of such pooled specimens would vary on different days. This fact would account for a fluctuation in QO_2 of independent experiments.

There appears to be a tendency for the treated semen to show a slightly lower rate of O_2 consumption. The pertinent data were tested by "Student's" method for paired observations. The value of Z obtained was only 0.275, giving the odds on significance of 3.25:1—

much below the accepted minumum for statistical significance. On the basis of these observations, therefore, a tentative conclusion can be reached that the irradiation of cock semen with 10,000 r. produces no effect on its QO_2.

As an interesting side light to the above tests, Figure 8 has been appended to indicate the relative rates of reaction in the two treatment groups, measurements being taken at 10-minute intervals. Although there is a steady slowing-down in the reaction rate, there is no material difference in this regard between the two groups.

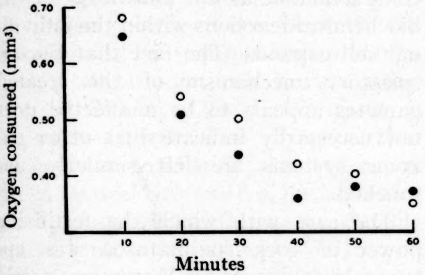

FIG. 8.—Rate of QO_2 reaction. ○ = control; ● = experimental.

EFFECT OF X-RAYS ON SUCCINIC DEHYDROGENASE SYSTEM IN COCK SPERMATOZOA

The next series of five experiments was designed to study the sensitivity of the enzyme system responsible for the oxidation of the succinic acid substrate. There is some suggestion that succinic dehydrogenase is adversely affected by the massive doses of X-rays (Havard, 1935). It was considered possible, therefore, that a parallel situation may exist in cock spermatozoa.

The semen was prepared for manometric tests in a manner similar to the plan used in QO_2 studies, with only a slight modification. Sodium succinate (0.1 cc. 0.1 M) was added to the main chamber of the vessel from the side arm following the accepted 10-minute equilibration period. The results of these tests are summarized in Table 9. The figures represent cubic millimeters of O_2 consumed in 60 minutes per milligram of dry weight.

Addition of sodium succinate to the normally present substrate in cock semen obviously exerts stimulating effect on the O_2 consumption of spermatozoa. The rise in the reaction rate was observed equally in the experimental and the control groups. It follows, therefore, that the enzymatic system responsible for this rise did not suffer from the exposure to 10,000 r.

TABLE 9

QO_2 OF IRRADIATED AND CONTROL SEMEN*

TEST NO.	IRRADIATED SERIES†		CONTROL SERIES	
	With Succinate	Without Succinate	With Succinate	Without Succinate
1	3.12	2.10	3.10	2.00
2	6.92	4.05	6.42	3.12
3	4.68	3.15	5.64	4.78
4	4.28	3.97	4.06	3.61
5	2.87	1.67	2.68	1.65

* Sodium succinate added to the substrate.

† Dose: 10,000 r.

EFFECT OF X-RAYS ON ANAEROBIC GLYCOLYSIS

The final series of experiments was concerned with anaerobic glycolysis of avian spermatozoa and its relationship to X-ray irradiation.

A sample of semen was made up to 5 cc. with Ringer's solution. To this suspension was then added 0.5 cc. of 0.154 M of $NaHCO_3$. This gave the pH of 7.4. The suspension was then saturated with the mixture of N_2 and CO_2 (95 and 5 per cent, respectively). Following this, 1 cc. of the suspension was poured into the main chamber of the vessel. The side bulb contained 0.1 cc. of glucose solution. After attaching the vessels to manometers, the gas mixture was again passed through the system for 3 minutes to drive off all traces of O_2. The first reading was taken while the glucose solution was still in the side arm. Following this, the latter was spilled over into the main chamber. Because of the vigorous nature of this reaction, larger vessels had to be used to maintain a closed system throughout the 60-minute observation period. Results are summarized in Table 10. The figures represent cubic millimeters of CO_2 evolved per milligram of dry weight.

It is obvious from the summary that, as far as the present series is concerned, the glycolytic function of the spermatozoa has not been affected one way or another by the application of 10,000 r of X-rays.

TABLE 10

ANAEROBIC GLYCOLYSIS BY IRRADIATED AND CONTROL SEMEN

Test No.	Irradiated*	Control
1	12.10	14.73
2	20.94	19.48
3	14.09	14.60
4	21.99	22.32
5	8.26	8.16

* Dose: 10,000 r.

DISCUSSION

Biological effects of X-rays, as studied in this investigation, roughly speaking, can be divided into two groups. One group is concerned with the modifications in the behavior and the functional activity of the irradiated spermatozoa; the other dealt with the developmental abnormalities of zygotes sired by the treated gametes. More specifically, the criteria used to study the X-ray–induced changes included (*a*) the degrees of fertilizing capacity of the spermatozoa following the application of X-rays of known intensity, (*b*) spermatozoan "longevity" in the reproductive tract of the female, (*c*) their motility and morphology *in vitro*, and (*d*) certain aspects of the gametic metabolism. Moreover, the F_1 generation provided some indirect information on the less apparent changes induced by X-rays in the paternal gamete.

These criteria were not of equal sensitivity. The basic function of the male gamete, i.e., its power to activate the ovum, was the first to suffer from irradiation. On the other hand, motility and metabolic activity were more resistant. There is no reason, of course, to conclude that the above list of criteria is exhaustive. Other, more refined approaches to the general problem will become available as our knowledge of the biochemical reactions within the individual cell expands. The fact that the respiratory mechanism of the treated gametes appears to be unaffected does not necessarily indicate that other enzyme systems are left similarly untouched.

The ease with which the fertilizing power of cock spermatozoa was apparently depressed by X-rays was somewhat surprising, in view of the resistance to this agent shown by the male gametes of *R. pipiens* in the work of Rugh (1939). Perhaps this involves the question of species differences. This is a plausible explanation when the work of Dalcq and Simon (1931) is taken into account. It will be recalled that these workers could not secure fertile ova of *R. fusca* when the male gametes were previously subjected to 3,000 r of X-rays. If such extreme differences can exist in the two species belonging to one genus, the phylogenetic distance separating amphibians and birds might well lead to even greater divergence in results.

The study of Henson (1942) is interesting in this regard, largely because the experimental material, the albino rat, represents a further rise on the phylogenetic tree. She irradiated male rats in the testicular region, to determine the effect of such treatment on the embryonic development of the resultant zygotes. Among other things, she found that out of the five matings attempted between male rats receiving 3,000 r and normal females, "two of the matings seemed to be fertile," leaving three, presumably infertile, to be accounted for. One can infer that the infertility of the latter was due to the irradiation treatment of spermatozoa. The fact that all radiations were performed on semen *in situ*, i.e., while still in the seminiferous tubules of the testicle, or in the epididymis, is unfortunate from the standpoint of drawing comparisons between the sensitivity of the semen in birds and rats. The *in situ* irradiation raises the question of dosality. The living tissue is known to exercise some screening effect on short-wave radiation; and it is, hence, problematical how much of the estimated 3,000 r applied to the testes actually reached the spermatozoa. As Dr. Henson points out, the *in vitro* irradiation of rat semen would be more preferable as a technique. However, this awaits further improvements in the methodology of artificial ejaculation in the rat.

Negative results obtained with the intraperitoneal type of insemination seem to indicate that the mere mechanics of the fertilizing process are not concerned here.

The question of the criteria used in identifying fertile blastodisks should be mentioned. Undoubtedly, the results would have been strengthened if it had been possible to subject each ovum to a detailed cytological examination or if some biochemical test were available enabling the investigator to classify ova as "fertiles" or "infertiles." However, an extensive series of observations made on the relative macroscopic and histological appearances of fertile and infertile blastodisks indicated close agreement between the two. The comparison showed that the eggs appearing "infertile" to the unaided eye show no signs of zygotic development when the blastodisk is examined histologically. The only possible difficulty may arise when dealing with the extremely early deaths.[2] To identify this condition, a combination of cytological, histological, biochemical, or/and biophysical methods may be required. It would not have been practical, however, to undertake cytological tests for fertility, because in the study of this type, where a good deal of the material is of a quantitative nature, there is a practical limit for delving into details. Little is known at present of biochemical or biophysical tests for the fertility of hen's ovum.

Increase in the lethality of the F_1 chick embryos seems to indicate that the X-ray hit on the spermatozoa does not necessarily incapacitate such cells from participating in fertilization. On the contrary, the existence of a fairly close degree of relationship between the intensity of radiation applied to the specimen and the proportion of lethal zygotes fathered by such semen clearly suggests that some of the "injured" male gametes can still function as activating agents. It is only beyond a certain critical level that one finds this capacity completely obliterated. Furthermore, it is problematical whether the extent of the gametic dam-

[2] Since this report was submitted for publication, considerable data have accumulated at this laboratory to indicate that, in the chicken embryos, such early deaths are of rather common occurrence among zygotes produced by the union of presumably normal gametes. On the basis of the evidence on hand (seasonal fluctuations within same families, etc.), it seems likely that this condition is largely caused by unfavorable environmental conditions which impinge upon the unshed ovum. The extent to which the short-wave radiation can modify this cause-and-effect relationship still remains an open question.

age, passed on to the zygote, is a function of the X-ray dose. The type of abnormality, though not their total frequency, is independent of the dose; abnormal embryos of various types were recorded throughout the biotic dose range adopted in this study. The phenomenon of reduced fertility and of increased mortality can perhaps be best accounted for by the "one-hit" type of action. This type of response (Henshaw, 1940) provides that the effects of X-rays are not cumulative. Rather, a single hit is sufficient to cause profound changes in the constitution of the affected gamete. In the present study the absence of cumulative effects, which are normally characterized by a sigmoid curve of response, justified the fitting of a straight line of regression to the fertility data, to indicate the probable slope of response (Fig. 1). This form of radiobiological reaction has often been reported in the literature on the subject, particularly with reference to induced mutations in *D. melanogaster* (Patterson and Muller, 1930; Schultz, 1936). The fact that the various types of teratological development in the experimental zygotes can be found over the entire range of doses studied does not weaken the original postulate regarding the type of relationship between the cause and the effect. It is possible that there are regions in the spermatozoon which possess varying degree of sensitivity to X-ray action. There is nothing new in this suggestion. Regional sensitivity differences to short-wave radiation which exist within an individual cell have been discussed by Delbrück (1940) and others. Applying this concept to cock spermatozoon, a hit in one locus may produce a totally different result from a hit in some other spot within the cell. If the spermatozoon, after receiving the hit, still remains capable of activating the egg, then the type of the subsequent development will depend on the location of the target area. It will be recalled that some zygotes ceased development very early in ontogeny, while others survived for a few days. Still others lived to hatch. Whether any of the embryos fathered by the "hit" sperm can survive the incubation period cannot be answered at present. Linear measurements of early embryos in the experimental group which appear normal to the eye showed that, as far as the gross over-all growth is concerned, they compare favorably with the corresponding measurements of the control embryos. Moreover, it has been shown that the chicks that hatch in the experimental group, as well as their control siblings, show comparable somatic growth rates. As will be pointed out later, it is possible that such embryos and chicks appear normal because they were fathered by the spermatozoa which had escaped the damaging effects of X-ray radiation. On the other hand, it is also possible that some of the chromosomal rearrangements resulting from hits do not produce any visible alterations in the pattern of embryonic and postnatal growth. Only proper genetic tests would then bring out their latent abnormality.

It must be admitted, however, that the criteria for "normality" or "abnormality" used in biological work are crude and, to some extent, subjective. Referring to the present study, undoubtedly many instances of abnormal ontogeny passed unnoticed because of their minuteness. Moreover, environment can easily obscure the picture. Certain nutritional deficiencies are known to cause early death or abnormal embryonic growth. Almost none of these teratological cases survive long enough to be tested genetically. But, assuming that the effect of environment is equalized throughout the series, the complexity of the living cell—in this case the spermatozoon, the functional activity of which is modified by X-rays—may result in a more or less incoherent series of changes under the hit-and-miss influence of Roentgen radiation.

The fact that the bulk, if not all, of the observed abnormalities occur early in prenatal life is not surprising. The constantly increasing complexity of the developing organism requires smooth adjustment of its component parts. Dunn (1941) points out that, while ontogenesis in its early stages may proceed uninterruptedly for some time in the absence of such a balance, the more exacting requirements of later ontogenesis conflict with the fundamentally unco-ordinated organismal complements. This brings the whole process to a more or less abrupt halt. Ephrussi (1935) found that bits of tissue, unable to grow *in situ* because of such fundamentally genetical incompatibilities, will thrive in tissue culture. This suggests that it is not the inherent inability of the individual cell to proliferate that is the factor, but rather the inability of such cells to co-ordinate their development into a coherent whole.

In live-chick blastoderms many of the studied abnormalities were characterized by the absence of differentiation beyond the formation of the first two germ layers. The appearance of chorda mesoderm is blocked; the situation is similar to that obtained by Grodzinski (1933, 1934) in the precooled fertile hen egg and observed in tailless rats by Chesley (1935) and Dunn (1941).

Some of the blastoderms went even further in this process of degradation, having apparently lost capacity for any differentiation above the level of elementary cellular division. In such blastoderms no entodermal constituents could be identified, indicating that gastrulation was completely blocked. Sonnenblick and Henshaw (1941) made a similar observation in a parallel situation in *Drosophila*. We do not know how long such chick blastoderms can live. The oldest, in terms of the recorded hours of incubation, was in the 120-hour group. In some respects such undifferentiated blastoderms can be likened to the actively proliferating tissue culture; both show a law order of differentiation.

Existing limitations in our powers of cytological analysis in the chicken prevent more searching approach to this problem. However, certain inferences, applicable to the present case, may perhaps be drawn from the study of Poulson (1940) on the effect of an unbalanced chromosomal complex in *Drosophila* on the developmental process of young zygotes. He found that the absence either of the whole or of a known portion of the *X*-chromosome leads to certain abnormalities in the subsequent ontogeny of the affected zygote. The absence of the entire chromosome produced a completely unorganized type of cellular proliferation. Such "embryos" eventually die. It should not be too speculative to draw a tentative parallel between these findings and the observations pertaining to embryonic abnormalities in the present investigation. Suggestion is therefore made that some of the observed abnormalities, particularly of the type which involves complete lack of organization, may be due to chromosomal loss occurring at the time of fusion of parental nuclei.

The majority of contemporary writers ascribe such teratological ontogenesis in the progeny of X-rayed parents to lethal mutations of one type or another produced by the treatment in the parental gametes. Hanson and Heys (1932) calculated that in *Drosophila* the application of 3,890 r of X-rays to breeding males produces 2.86 per cent of sex-linked lethal mutations. Whiting (1940) quotes the figure of 10,000 r as the threshold level, beyond which dominant lethal "change of some sort" is produced in almost all irradiated gametes. This interpretation of the phenomenon of zygotic lethality fits, in broad terms, the situation observed in the X-rayed cock spermatozoa. There is no information on hand, however, to show whether the rise in mortality rate is due to gene mutations (i.e., chemical changes within the gene) or is merely an outcome of structural changes in the chromosome complex of the individual gamete. Snell and Ames (1939) explained their results in mice on the basis of induced structural changes in chromosomes.

Additional evidence that X-ray radiation acts through the chromatin material of the irradiated male gamete is supplied by the data bearing on the secondary sex ratio.

As already indicated, there was no attempt made in this study to make any computations of sex ratios before hatching. A study by Byerly and Jull (1935), based on 17,989 chick embryos which had died between the ninth and the twenty-first day of incubation, showed the sex ratio to be 47.56. This would result in an excess of males at hatching time. Landauer and Landauer (1931), on the other hand, reported the secondary ratio of 48.77, i.e., a deficiency of males at hatching. They arrived at this figure after examining 67,993 live-born chicks. In both these cases, deviations from the theoretical 50 per cent ratio are statistically significant. The existent disagreement on the subject prompted Crew (1938) to suggest that "different 'breeds' yield different sex ratios." The relevant data collected in the course of this investigation, though limited in scope, offer an opportunity to observe the effect of radiation treatment on the secondary sex ratio of the group of chicks which are comparable in all respects but one (that of treatment) to another group of chicks. The two classes are, broadly speaking, genetically similar, thus providing a critical basis for comparison.

The fact that the secondary sex ratio in the X-ray radiated group has been altered is quite suggestive. If it is accepted that, in the higher forms at least, sex determination is subject to genic control, then the theory on altered sex ratio formulated by Haldane (1922) and Lenz (1923) may be applicable to the present case. According to the theory, the presence of lethal or of semilethal sex-linked factors in a population will largely affect the heterogametic sex. In the homogametic sex the factors involved would have to be in homozygous condition to assert themselves. On the basis of this theory, then, one would expect more female chick embryos to die if the radiation treatment of the male gametes produces recessive lethal or semilethal mutations involving sex chromosomes.

Perhaps the most interesting single fact to come out of this study is the absence of Hertwig phenomenon, which has been found to appear in a variety of unrelated forms. If one accepts his explanation of the somewhat unexpected behavior of the heavily radiated frog spermatozoa, then the cock spermatozoa show a peculiar form of the all-or-none type of response. Thus, the "hit" spermatozoa apparently are capable of doing two things: either they lose their fertilizing capacity altogether, i.e., when the injury is too great, or they accomplish fertilization in a normal fashion, with the chromatin material of the two parents taking part in directing zygotic development. At present the degree of normality is of no concern to us. There is no evidence to indicate that the hen ovum is capable of parthenogenetic development; and, until such is forthcoming, one is forced to assume that none of the observed abnormalities represent parthenogenesis. The fact that the lethality of the embryos rises with the intensity of X-rays adds further weight to this interpretation.

When dealing with the activating agent, represented in this case by the spermatozoon multiplied millions of times, one cannot altogether overlook the phenomenon of "shielding." The fact that all of the chicks, which have survived to hatching time, appear to be normal, suggests two explanations. One is that the chicks were sired by spermatozoa which, through shielding, had managed to escape biologically demonstrable injuries produced by X-rays. The existence of this type of mass action in the response of spermatozoa to short-wave radiation has been discussed by Allee (1931). The 100 per cent lethality among zygotes sired by spermatozoa exposed to the maximum biotic dose would merely indicate, then, that the intensity of the treatment was so great as to overcome the protective shielding effect. The other possibility is that the chicks were sired by spermatozoa which, by chance, had escaped X-ray hits. Again the damaging effect of radiation will be proportional to dosage: the higher the dose the greater the volume of ionization produced, leading to a situation in which all or nearly all gametes have been either directly or indirectly (beta particles) hit.

A number of questions raised in this study are left unanswered. There is little doubt that the prime factor or factors responsible for increased lethality of the experimental zygotes is located in the nucleus of the radiated paternal gamete. The spermatozoon is almost a naked nucleus provided with powers of locomotion. It is difficult to assign any role in the process of X-ray–induced modifications to the small amount of cytoplasm present in the male gamete. However, even if a plausible conclusion can be drawn with respect to the site responsible for these changes, nothing is known about the exact nature of such changes.

Even more perplexing is the situation with regard to the damaging influence of radiation on the fertilizing capacity of the spermatozoan. Tests showed that the *in vitro* motility is not affected within the biotic range of radiation. No evidence is available on the motility behavior pattern of the treated gametes in the oviduct. Indirect evidence, supplied by the fertility data, indicates that the irradiated spermatozoa reach the shed ova as quickly as the controls do.

The bulk of pertinent evidence indicates that there is a latent period in the response of the cell to short-wave radiation. Applying this fact to the radiobiological reaction within the spermatozoon, it is reasonable to expect that the physiological mechanism governing motility is not sufficiently altered between the time the semen specimen is irradiated and inseminated and the time the gametes reach the upper end of the oviduct. Mimura (1939) has shown that the chicken spermatozoon covers the distance from the uterine portion of the oviduct to its upper region, where fertilization takes place, in less than 30 minutes. Moreover, Parker (1931) indicated that spermatozoa in the pigeon do not depend entirely on their power of locomotion to reach the upper portions of the oviduct. Therefore, it is reasonable to conclude that the motility of the irradiated spermatozoa is not a factor responsible for the progressive drop in their fertilizing capacity.

Motility of the spermatozoon is an expression of certain, at present little understood, biochemical activities within the protoplasm of the cell. Lardy and Phillips (1941) have shown that in the bull spermatozoa the glycolytic mechanism provides the energy which sustains motility. It is quite probable that the same type of relationship between glycolysis and motility exists in cock spermatozoa. The fact that X-ray radiation does not affect the rate of glucose utilization in the treated cells with motility also remaining unaffected tends to corroborate this assumption.

Longevity, i.e., the ability of the irradiated spermatozoa to survive in the reproductive tract of the female, also apparently is unaffected by the treatment.

So we see, on one hand, a decided drop in the proportion of the ova fertilized by the treated spermatozoa and, on the other, the apparent "normality" in the behavior of such spermatozoa. How are these two facts to be reconciled? It has been shown on numerous occasions (Munro, 1938, and others) that fertilization following insemination, other factors being equal, depends upon a certain concentration of spermatozoa. The mere presence of relatively few spermatozoa among great masses of functionally inert germ cells will not assure successful fertilization. In the chicken, for example, some hundreds of thousands of active spermatozoa are necessary to insure fertilization of a single ova. If the irradiation treatment produces irreversible and unfavorable changes in the physiology of the spermatozoon, changes which cannot be gauged by such relatively crude detectors as motility or morphology, then such cells will become incapable of activating the ova. Numerical relationship between functionally active and functionally inactive gametes will then determine the proportion of eggs fertilized by the specimen. When the dose is sufficiently high, there are few, if any, uninjured spermatozoa. Such cells will be thoroughly "diluted" in the mass of functionally inert spermatozoa. Fertility will then become nil.

SUMMARY

1. The .fertilizing capacity of cock spermatozoa, as indicated by the available methods of detection, is adversely affected by the soft type of X-ray irradiation. It is completely lost between 5,544 r and 6,488 r.

2. The inverse type of relationship between the fertilizing capacity and the X-ray dose can be expressed by a straight line of regression. In the present case the equation for the line was calculated to be $25-0.3268y$. The coefficient of correlation between the two variables is -0.9631.

3. The type of fertilization is not responsible for the reduced fertilizing power of treated spermatozoa.

4. The injurious effect of X-ray radiation is meted out directly to the spermatozoa and is not mediated via the fluid portion of the semen. The pH of the irradiated semen is unaffected by the treatment.

5. During the first 10 days following irradiation, longevity of the treated spermatozoa compares favorably with that of controls. The total functional life-span, however, is shortened.

6. Motility and morphology of the treated spermatozoa are not altered within the biotic range of radiation. They are modified, however, by more intensive treatment.

7. The X-ray treatment of male gametes results in a preponderance of male chicks at hatching time from zygotes fathered by such gametes.

8. Zygotic mortality is closely related to the intensity of radiation applied to the paternal germ cells. The bulk of deaths occurs during the early part of the incubation period.

9. Lethality of the embryos fathered by the irradiated spermatozoa is associated with increased frequency of abnormal development.

10. Irradiation of spermatozoa with 10,000 r has no detectable effect on their Qo_2, Qco_2, and succinic dehydrogenase system.

11. It is concluded that the irradiation of cock spermatozoa with doses as high as 152,000 r does not produce Hertwig phenomenon in the chicken ova exposed to such spermatozoa. The reduced fertilizing capacity is due to the increasing proportion of injured germ cells with the rising intensity of irradiation. This "dilution" of still functional gametes with functionally inert, though motile, spermatozoa eventually leads to the complete loss of fertilizing capacity by the specimen.

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SOME MEASURABLE EFFECTS OF SOCIAL ORGANIZATION IN FLOCKS OF HENS[1]

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MODERN studies of social hierarchies among birds date from the work of Schjelderup-Ebbe (1922). The field has developed rapidly, and summaries of progress have been fairly frequent (e.g., Allee, 1942, 1943; Collias, 1944). There are many indications that high social rank may be associated with definite survival value for individuals near or at the top of the social order, as contrasted especially with those near or at the bottom of the given hierarchy.

The possible correlation between individual social status and individual survival is relatively easy to investigate. It is fully as important, though more difficult, to inquire into the survival values associated with organized, as compared with unorganized, groups. We have not yet had access to material appropriate for testing exactly this point or to sufficient space in which to conduct a simple, diagrammatic experiment along somewhat related lines as follows: Using space available to us, we have tried to measure various effects produced when mature hens are allowed to establish and maintain a peck-order in flocks composed of the same individuals, as compared with flocks that are made to undergo constant reorganization and also as compared with isolated hens. Under the conditions of our experiment the organized flocks show group-survival values which, if they occurred in nature, might afford a basis for natural selection. Two preliminary statements have preceded the present definitive report (Allee and Guhl, 1942; Allee, 1943).

ANIMALS USED AND THEIR TREATMENT

White Leghorn hens from Indiana Farm Bureau stock were used exclusively; they had been intraflock bred since 1932. The individuals were marked with appropriate leg bands immediately upon receipt, on June 17, 1942. They were near the end of their first laying season and, except for having bare backs, were in good plumage and in excellent physical condition. The hens were separated at random, as follows: Seven were placed in each of four pens in the poultry-house at Whitman Laboratory, the only henhouse space available for our work; and twenty-one more were isolated in individual cages in a near-by green house. The flocks in three of the four pens were allowed to establish and maintain a peck-order and served as controls. The fourth pen, situated between control flocks, housed the experimental flock in which, for the first week, one of the original residents was removed and replaced daily by a hen from isolation, and thereafter the oldest resident was similarly replaced

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every day during rounds I–III and every other day during rounds IV–VI. These and other details of procedure are summarized in Table 1.

TABLE 1

SUMMARY OF PROCEDURE

Rounds	Weeks Ending	Weeks	Food			Alternates		Control Flocks
			Composition	Time (Twice Daily)		Alternations	Time in Pen (Days)	
				Pens (Minutes)	Isolation			
I-II.....	6/24–8/12	1–8	1/3 mash; 2/3 grain	6/18–7/8 30 7/9–8/12 60	No records	From isolation	7	W, G, and P
III......	9/4 –9/25	11–14	Same	60	120 min.	From isolation; same order of rotation	7	Same
IV-V....	10/2 –1/15	15–30	Same	60	120 min.	From isolation; order changed	14	Same
VI.....	1/22–3/12	31–38	Same	90	120 min.	Same	14	Same
Adjustment week for selected group	3/19	39	Same	90	120 min.	....	......	Same
VII.....	3/26–5/14	40–47	Same	90	90 min.; began (42d week)	Rotated from pen to pen	14	W only
VIII....	5/21–7/9	48–55	Mash all day; grain 60 min. twice daily	....	Mash all day; grain 60 min. twice daily	Same	14	Same

The word "round" is used here to mean the time taken before the experimental flock is again made up of the original individuals; each has meantime spent its appropriate period in isolation. In the following account, individuals will be referred to as "controls" when they belong to unchanged flocks; "alternates" when in the experimental flock or flocks; and "isolates" when in isolation. So-called "experimentals" include both alternates and isolates.

Isolation for even 21 days caused returned alternates to meet former penmates as strangers and to be treated by them in the same way. They were attacked on entrance into the experimental pen and the peck-order was in a continual state of flux. During the first three rounds the birds were transferred from isolation to the experimental flock in the same order; for the next three rounds the order was changed to give each hen the opportunity to meet each of the other birds of the alternate-isolate group while in the experimental pen. This change in the order of admission to the pen resulted in some inequality in the number of consecutive days spent in isolation.

The pens used have been described elsewhere (Allee, Collias, and Lutherman, 1939); they are four in number and are 14.5 feet long, 4.5–5.0 feet wide, and about 8 feet high. They are illustrated in Figure 1. Since this is the only poultry-house space that has ever been available for our work on social order in *Gallus domesticus*, the isolated hens were housed in a partially darkened greenhouse in pens with a floor area approximately one-seventh of that available for each flock; the dimensions were: length, 4 feet; width, 2.3 feet; height, 2.5 feet. Three sides were made of wallboard, arranged so that the birds could not see each other; the fourth side and top were of common chicken wire. The general relations are shown in Figure 2. These isolation pens were near an inside partition, the lower part of which was made of wood; they were roofed over by tar paper at about the height of the henhouse ceiling. The inner roof served to reduce the light intensity to approximately that of the regular pens and also protected the hens from the leaky greenhouse roof both during rains and on hot days in summer, when the glass roof was sprayed to help lower the greenhouse temperature.

FIG. 1.—A flock in one of the pens used in these experiments. Photograph by K. Toda.

Both the greenhouse and the poultry-house were heated, and the temperatures were under approximate control during the winter. It was necessary, for reasons not connected with this experiment, to maintain a winter temperature of 75° F. in the greenhouse; accordingly, the poultry-house temperature was raised to about 65° in an effort to maintain no greater temperature difference than 10°; usually the temperature difference was much less. The poultry-house was relatively warmer in summer and cooler in winter than the greenhouse.

The chicken-house pens have large south windows, and light measurements indicate that on overcast days (common in Chicago) the hens housed there received natural daylight for a period that was intermediate between that occurring in the lightest and the darkest isolation pens in the greenhouse. On bright, sunny days the photoperiod in the henhouse about equaled that in the darkest greenhouse pens. Daylight was supplemented in both houses during much of the year by artificial lights that were automatically turned on at 7:00 A.M. and off at 5:30 P.M. "daylight-saving" time over the isolation pens, and with similar hours in the henhouse except that there the added light was automatically turned off between 11:30 A.M. and 1:30 P.M. Shaded 25-watt lamps at approximately the same elevation were used throughout; they delivered about 5 foot-candles of light on the floor area directly below the lamps.

Although differences in housing were kept at the lowest possible minimum under available conditions, they may well have affected the experimental findings, since the isolated hens were regularly transferred to the experimental flock and since there was probably some lag in the environmental adjustment. Accordingly, in order to reduce recognized environmental differences, after 38 weeks of experimentation the procedure was changed for the final two rounds, as follows: Among the three control flocks, one was selected to continue as a single control group. This flock had had no replacements; its food consumption and body weights approximated the average for all controls; its pecking frequency was above, and its egg-laying was below, the control average. These last two items were thought to be of decided importance, and the continuing control flock was so selected as to minimize observed differences between controls and alternates in an attempt to make the final test more critical.

The other two control flocks were taken over directly into the new experimental routine, and the third experimental group was composed of seven hens chosen from the twenty-eight alternates and isolates to give a functional cross-section. More precisely, these experimental birds were divided into seven frequency classes on the basis of the number of pecks each had delivered during the preceding six rounds. One hen was selected from each frequency class after consideration of the social status she had attained while a member of the flock of alternates, relative intensity of pecking, avoidance of other hens, general activity, and comb size. In egg-laying the new flock ranked with the mean of all isolates at that period of the year (see Fig. 6).

No alternations were made during the thirty-ninth week, in order to permit the newly selected alternates time to form a peck-order among themselves. Thereafter, every second day one hen was transferred in a regular order from each of these three experimental pens to another one, with a resulting slow rotation of membership among these three flocks. The remaining twenty-one hens were left in isolation in the partially darkened greenhouse throughout the seventh and eighth rounds. Under this modification of the original experiment the new alternates were housed, without variation, under conditions that closely approximated those of the controls, thus simplifying the experimental situation. To reduce the frequency of hens meeting former penmates before less than 14 days of separation, the hens of a given pen were distributed between the other two experimental pens as the fortnight-long residence of each was concluded. Under both the earlier and this last arrangement, the unchanged, organized flocks could be compared with associated flocks that were never permitted to become fully organized.

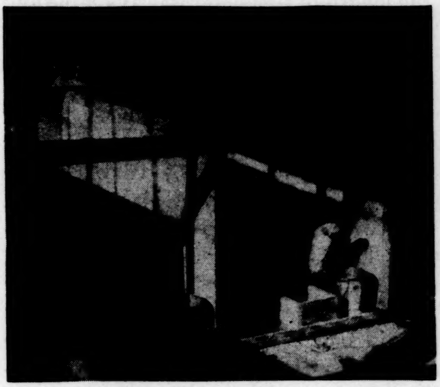

FIG. 2.—Hens in isolation. Photograph by K. Toda.

Twice daily, all birds were fed a mixture of about one-third mash and two-thirds scratch grain. High-grade laying mash and mixed grains were obtained from a reliable commercial source. Those in flocks were fed from a metal box 12 × 10.5 × 5 inches with a 1.5-inch lip to lessen spillage. About, 1,800 gm. of the mixture were placed daily in the food trough of each pen, and the food consumed was determined by weighings. Isolates were given about 300 gm. each, in small heavy wooden boxes; and their daily consumption was also ascertained, beginning with the tenth week. More food was made available than was eaten during the time allotted to feeding.

The small size of the food trough increased the competition between individuals in experimental and control flocks; the basic appetite, modified by the length of time food was available and by social conditions, determined how much food the flock consumed. The aim was to adjust feeding time so as to insure a constant maintenance of body weight and egg production for the controls; this had to be determined by trial. For the first 3 weeks, food was placed in each pen twice daily for 30 minutes. The controls lost weight under this treatment, and the length of possible feeding time was doubled. Time of exposure to food was increased again to 90 minutes twice daily after the thirtieth week, in an effort to reduce the pecking frequency of the controls. During the final round of the experiment, mash was available all day, and grain was fed at observation periods twice daily for 60 minutes each time. This terminal treatment brought the feeding schedule close to customary methods of poultry husbandry. These and other details are summarized in Table 1.

Since the isolates lost weight when present in the experimental flock as alternates, they were fed longer than the penned flocks during the first 39 weeks, in order to permit them to regain weight before being again placed with their fellows. Beginning with the forty-second week, both penned and isolated birds were fed for the same length of time, for comparative purposes.

Sundays excepted, each flock was under direct observation during feeding time for 30 minutes each week-day morning and again in late afternoon. The feeding order of the pens was rotated daily. Pecks and threats were observed in order to determine peck-orders and as a measure of social activity. A grand total of over 230,000 pecks and threats made it possible to make analyses with some confidence. A peck or threat was recorded without distinction whenever there was a definite domination-subordination reaction between members of a temporary contact or reaction pair.

Controls and alternates were weighed at the end of each experimental week. The greatest length and the height of the point of the comb over the eye were measured in millimeters at this time. The isolates were also weighed and measured when taken from and returned to isolation.

Trap nests gave a satisfactory egg record for individuals; only about 3 per cent of the eggs were unplaced. All records were summarized weekly and again at the end of each complete rotation (round) of the twenty-eight experimentals. The experiment lasted from June 17, 1942, to July 10, 1943—fifty-five weeks, eight experimental rounds. No records were taken during two vacation weeks (the ninth and tenth), and no alternations were made during the thirty-ninth week. Pecks were not recorded for Sundays, though the birds were alternated on that day and otherwise received their usual care. The weekly means are based on a 7-day week except for pecking, for which 6 days of records are averaged.

Eleven hens died during the experiment; four of these were controls, and seven were experimentals. Of the deaths among the controls, two occurred during the second week and one each during the thirty-fourth and the fifty-fourth week (rounds I, VI, and VIII, respectively). One of the experimentals died in each of rounds IV, V, and VI; and two in each of the last two rounds. These hens were replaced by others taken from the original commercial flock. Replacements were made at the stage of the experiment when they would be the least upsetting to the general progress of the experiment. The effects of replacements were negligible among the experimentals, as this group was undergoing constant reorganization. On the other hand, the introduction of a stranger into a control flock was disruptive and made the social conditions temporarily approach those in the flock or flocks of rotating alternates.

All feeding and care of the birds and their pens (except for the ninth and tenth weeks) and all recorded observations were made by the first author. He was near the birds several hours each day and was able to reduce extraneous disturbances to a minimum.

The state of well-being of the flocks may be judged from the observation that the hens in the control flocks and the isolates produced an average of 122 eggs per individual during the 51 weeks from mid-June, 1942, to mid-June, 1943. In order to treat all hens in as nearly uniform a manner as possible, individuals from the control flocks were handled as often and as much as were the alternates in transferring them from isolation to the experimental pen.

When it is recalled that our hens (*a*) had relatively low laying records as compared with others in their original farm flock, (*b*) were in their second year of production, and (*c*) were subjected to the handling just mentioned and that needed for weekly weighing and measurement of comb, egg production of controls and isolates seems to be as high as could reasonably be expected.

OBSERVATIONS

The twenty-one control hens and the twenty-eight experimentals (alternates plus isolates) were separated by random selection. The two subpopulations so established did not differ significantly in any observed or measured characters, as revealed either by measurements at separation or by performance in the early weeks of the experiment, when the control flocks were establishing their social hierarchies and when all were becoming adjusted to new conditions. These first results are summarized in Table 2.

TABLE 2

MEAN INITIAL MEASUREMENTS AND MEAN WEEKLY PERFORMANCE DURING THE FIRST 8 WEEKS

COMPARISONS OF MEANS	MEAN INITIAL MEASUREMENTS			P-VALUE	
	Controls	Experimentals	Difference	By Population	By Weeks
Weight (gm.)	1,919	1,898	21	0.76	......
Comb: length plus height (mm.)	133	136	− 3.0	0.36	......
Pecks	172	177	5	0.9	0.56
Eggs	2.6	2.5	0.1	0.8	0.77
Change in weight (gm.)	−35	−65	30	0.1	0.08
Food consumed (gm.)	495	423	71	*	0.02

* No individual records.

Unless otherwise stated, estimates of statistical significance are given throughout this report, as in Table 2, in terms of probability (*P*). This is calculated by obtaining the value for *t* from formulas (1) and (2).[3]

$$t=\left.\frac{\bar{x}_1-\bar{x}_2}{\sqrt{\dfrac{[(\epsilon x_1^2-\bar{x}_1\epsilon x_1)+(\epsilon x_2^2-\bar{x}_2\epsilon x_2)]\left(\dfrac{1}{n_1}+\dfrac{1}{n_2}\right)}{n_1+n_2-2}}}\right\} \quad (1)$$

---

[3] Or by a simple modification of these formulas.

where $\bar{x}_1$ is the mean of observations for one population; x_1 represents each individual value; n_1 gives the number of x_1 available for study; and $\bar{x}_2$, x_2, and n_2 give similar values for the second population.

After calculating t, P is found by consulting a table of probabilities for the distribution of "Student." With simple paired comparisons this formula becomes that given in equation (2):

$$t = \frac{\bar{x}}{\sqrt{\frac{\epsilon x^2 - \bar{x}\epsilon x}{n(n-1)}}} \qquad (2)$$

P-values become worthy of being taken seriously when they equal or are less than 0.05 (5×10^{-2}); the smaller the fraction, the higher the statistical probability. A P-value of 0.05 is the equivalent of having the observed difference of two means equal to twice the standard error of the mean difference or three times the probable error.

There are two relatively simple methods available for the statistical analysis of results such as we are presenting: (*a*) the variations may be examined in terms of the individuals present, an analysis by compared populations; or (*b*) the succession of weekly mean differences may be examined to find their variation and so discover the steadiness of performance, as determined by the degree of fluctuation from week to week. When possible, both methods will be used throughout this report. The former is generally the more conservative, but it may not tell the whole story in the case of rapid changes with seniority in the flock of alternates (p. 338) and when there are marked seasonal trends, as in egg-laying. In the latter instance the week-by-week difference in performance of controls and experimentals may have real meaning despite a steady change in the position of the weekly means.

As shown in Table 2, the difference in food consumption between the flocks of control hens and the alternates, when analyzed by weeks, furnishes the only apparent exception to the statement that the two artificially divided flocks were initially parts of a single population. An inspection of Figure 4 shows that the control flocks actually consumed less food than did the alternates during the first week, but they soon established the trend toward a higher level of food consumption. The greater amount of food eaten by the control hens, as shown in Table 2, seems, therefore, to be an expression of an early differentiation rather than of an innate difference between experimental and control flocks.

Having established the original unity of the stock in hand, we will now consider the effect of continual readjustment in the flock of alternates on the following activities: (*a*) social activity, as shown by the amount of pecking; (*b*) food consumption; (*c*) maintenance of body weight; (*d*) rate of egg production; (*e*) size of comb; and (*f*) seniority and social status.

FREQUENCY OF PECKING

The amount of social interaction is indicated by the data summarized in Table 3 and Figure 3. The data record the number of pecks or threats delivered. It will be well to examine first the trends shown in Figure 3. After the first week, in which the opposite was true, and with a few other scattered exceptions, the socially more stable controls almost always pecked less than did the alternates. The occurrence of pecking was relatively high in both sets of birds in the initial weeks and remained at the same high level among the alternates, or even increased in frequency until the last 8 weeks (period E), when mash was before the birds all day. Under these conditions the rate of pecking among the alternates

TABLE 3

SUMMARY OF PECKS AND THREATS DELIVERED PER BIRD PER WEEK

Rounds	Weeks	No. of Pecks and Threats			*P*-Values*	
		Controls	Alternatives	Difference	By Population	By Weeks
I–II........	1 – 8	172	177	5	....	....
IV–VI.......	15–38	133	209	76	1×10^{-2}	2×10^{-4}
III–VI......	11–38	132	200	68	6×10^{-5}	1×10^{-7}
VII.........	40–47	201	221	20	....	....
VIII........	48–55	70	138	68	4×10^{-2}	2×10^{-4}
VII–VIII....	40–55	136	180	44	....	4×10^{-3}
I–VIII......	1 –55	139	191	52		2×10^{-7}

* Dots show that the *P*-values are greater than 1×10^{-1} and therefore are fairly remote from statistical significance.

fell to about the level found in the control flocks in winter, when they were on a restricted feeding schedule.

As the organization became fairly stable after the tenth week, pecking among the controls fell from an initial weekly mean of 172 pecks per hen to an average of 132 during weeks 11–38, inclusive. The rise to 201 during round VII (Table 3) is more apparent than real, since the most active and erratic of the three control flocks was kept as the sole control for this period. The final decline to a mean of only 70 pecks per bird per week came with the presence of mash throughout the day and is to be contrasted with a mean of 138 for alternates under the same final conditions.

The statistical analyses show the following relationships between controls and alternates: (*a*) lack of statistical significance during the first 8 weeks; (*b*) high statistical probability during rounds III or IV to VI; (*c*) low probability in round VII, probably because of the erratic behavior of the single control flock; (*d*) good probability when analyzed by weeks for round VIII and when rounds VII and VIII are considered together; and (*e*) very high statistical probability when the complete series of weekly differences are compared by weeks.

In general, the hens in the organized flocks pecked each other less than did those in the flock or flocks that were held in a constant state of reorganization.

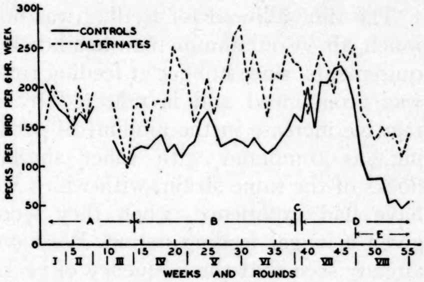

FIG. 3.—The average number of pecks delivered each week based on pecks recorded during observation periods totaling 6 hours per pen per week. Alternates were rotated daily during period A and every second day in B. No changes were made in the week labeled C; and during D the rotations were made on alternate days between three experimental flocks rather than between one experimental flock and the birds in isolation, as in A and B. Mash was present all day in E. (See Table 3.)

FOOD CONSUMPTION

In considering these results on pecking activity and in the discussion of food consumption to follow immediately, it may be well to keep in mind that both controls and alternates were restricted as to length of feeding time during most of the entire experimental span. Apart from this limitation, their treatment and housing compared favorably with accepted methods of good poultry management. Titus (1928) found that White Leghorn hens require, to maintain body weight, about 65 gm. per day of food somewhat similar to that which we used, and an additional 40 gm. for each egg laid. Brody, Funk, and Kempster (1938) found that their flocks required 69 gm. daily to maintain weight and 55.4 gm. additional food for each egg produced. Study of the data to be presented concerning food consumed and the number of eggs laid by our control flocks will suggest that, with the exception of the first two rounds, these hens were held at or near the threshold of food requirements for maintenance of body weight at the observed level of egg production.

The time allowed for feeding was not much above minimum maintenance requirements, since hunger at feeding time was pronounced and is reflected by a relative increase in the amount of pecking, as compared with other similar flocks of the same strain, with which we have had experience, when they were given a longer feeding period. We have already seen that the frequency of pecking decreased in the eighth round when food was available all day. The generally increased amount of fighting produced a relatively high number of reversals in the peck-right behavior of individuals and a development of pecking triangles in these control flocks during the early part of the experiment.

Weekly means of total food eaten are shown in Table 4 and Figure 4. Since we could not determine the amount eaten by each hen, the statistical analysis can be given only in terms of the deviations recorded week by week. Features to be noted include the following: After an initial week at about the same level of food consumption as the alternates, the control flocks rapidly increased their rate of food intake to approximately double the amount eaten during their first week of adjustment to a totally new physical environment and to close contacts with these particular flockmates. The average amount eaten did not fluctuate as widely as among the alternates; and during weeks 11–38, inclusive, the weekly mean was 583 gm. per hen. Food consumption did not increase, except temporarily, when feeding time was raised from a total of 2 up to 3 hours per day. A decided increase came with still longer feeding time at the height of the laying season. Other smaller and shorter apparent trends of some weeks' duration may or may not be important; they will not be considered further. The apparent increase in food consumption for the controls in round VII is not statistically significant when compared with the amount eaten by this control flock in round VI.

The alternates also increased their rate of food consumption as they became accustomed to their new surroundings. They leveled off at an average decidedly below that shown by the organized control flocks; and the mean difference, which is on the order of 150 gm. per hen per week during rounds III or IV through VI, has high statistical probability. In general, the alternates followed somewhat the same major feeding trends shown by the control flocks but at a lower level of food consumption. As concerns quality and quantity of food and the time allotted for its consumption, they were fed similarly at all times.

Exact determinations of food consumed by the isolated hens were not begun until more important procedures became routine. Data are lacking for the first 10 weeks. In general, the amount of food eaten by the isolates was of the same order of magnitude as that taken by the

TABLE 4

SUMMARY OF WEEKLY MEANS OF FOOD CONSUMED PER BIRD PER WEEK

ROUNDS	WEEKS	MEAN GRAMS PER WEEK			*P*-VALUES BY STATISTICAL ANALYSIS* (BY WEEKS)		
		Controls	Alternates	Isolates	Controls versus Alternates	Controls versus Isolates	Isolates versus Alternates
I-II	1 - 8	495	423	....	2×10^{-2}	....	....
IV-VI	15-38	586	432	580	2×10^{-4}	....	2×10^{-4}
III-VI	11-38	583	438	580†	$<1\times10^{-7}$	....	1×10^{-7}
VII	40-47	614	490	634	$<1\times10^{-7}$	....	$<1\times10^{-7}$
VIII	48-55	750	594	646	$<1\times10^{-7}$	1×10^{-2}	....
VII-VIII	40-55	682	542	640	$<1\times10^{-7}$	3×10^{-2}	5×10^{-3}
I-VIII	1 -55	600	467	....	2×10^{-7}	....	2×10^{-7}

* Where no figures are given, the *P*-values are greater than 1×10^{-1}; they must be 5×10^{-2} or less to be taken seriously.

† Twenty-seven weeks.

control flocks. Only during the final round, when feeding conditions were equalized (period G of Fig. 4), was their food consumption significantly different from that of the controls. The general seasonal trends in food consumed by these isolates suggest a positive correlation with seasonal trends in the laying cycle.

Until this final round the isolated hens had food before them for a total of 4 hours a day. During the first 4 weeks this was four times the length of time that control and alternating flocks had food available. Thereafter, through week 30, the isolates could have eaten twice as long as did the other hens; during weeks 31 through 47 food was available for 4 hours each day, while the controls and alternates could only eat at most 3 hours daily. In the last round all were treated alike with respect to the amount of time food was available.

As might be expected, the difference between the amount eaten by isolates and alternates is of the same order of magnitude as, and of statistical probability similar to, that between alternates and controls. Apparently, the lessened food consumption by the alternates is one consequence of increased social tension, as measured by increased pecking; and this, in turn, is associated with the steadily recurring introduction of new hens. Apparently, too, either the control hens ate to satiety under routine conditions—a suggestion of doubtful validity, in view of their increase in food intake when mash was made available throughout the day (round VIII)—or the isolated birds ate less than they might have done if they had been stimulated by seeing others eat. Such social stimulation

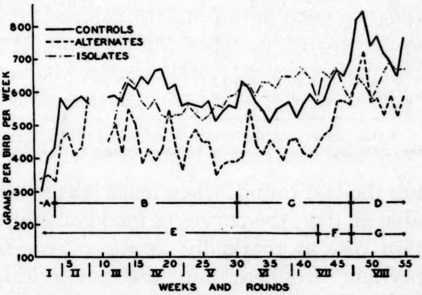

FIG. 4.—Average grams of food consumed per bird per week. During periods A, B, C, and D, controls and alternates were fed twice daily for 30, 60, 90, and 60 minutes, respectively. In D, mash was present all day, in addition to the grain fed at the time of observation. Isolates were fed for 120 minutes in E, for 90 minutes in F. During G they received the same feeding treatment as the other birds.

to increased feeding is known for other animals (Bayer, 1929, for the domestic fowl; Harlow, 1932, for rats; and Welty, 1934, for fish). Our isolated hens could hear noises made by their neighbors but could not see them; apparently, visual stimuli were needed to attain maximum food consumption.

Comparisons between Tables 3 and 4 and Figures 3 and 4 indicate an inverse relationship between frequency of pecking and the amount of food eaten. During the last round, when mash was available all day, the curves of food consumption rose as markedly as the curves of pecking dropped, and the nonpecking isolates did not show a comparable trend. As we shall see shortly, this relationship of relative levels of aggressive social interaction to avian economics is reflected in biological success, as expressed in weight and in egg production.

TABLE 5

SUMMARY OF WEEKLY MEANS OF CHANGES IN BODY WEIGHT

ROUNDS	WEEKS	MEAN CHANGE IN GRAMS		*P*-VALUES BY STATISTICAL ANALYSIS*	
				By Populations	By Weeks
		Controls	Alternates	Controls versus Alternates	Controls versus Alternates
I–II	1– 8	−35	−63	1×10^{-1}	8×10^{-2}
IV–VI	15–38	+ 6	−37	6×10^{-6}	2×10^{-4}
III–VI	11–38	+ 7	−40	6×10^{-7}	4×10^{-5}
VII	40–47	+ 1	−24	2×10^{-2}	8×10^{-2}
VIII	48–55	+27	+16	....	....
VII–VIII	40–55	+14	− 4	6×10^{-3}	....
I–VIII	1–55	+ 3	−33		4×10^{-6}

* Dots show that the *P*-values are greater than 1×10^{-1}; they must be 5×10^{-2} or less to be taken seriously.

MAINTENANCE OF BODY WEIGHT

The average gain or loss in body weight per bird per week is summarized in Table 5 and Figure 5. The data show, as might be expected from the discussion of food consumption, that the control flocks tended to maintain their weight during the period from week 11 to week 47, inclusive. On the other hand, the socially unstable alternates were much more erratic in weight maintenance. As in the preceding figures, the means are plotted in Figure 5 without smoothing and show, in part, the effect of the constantly shifting experimental population. Not only was there an increased frequency of pecking, but the physiological characteristics of the experimental flock varied with the individuals that composed it.

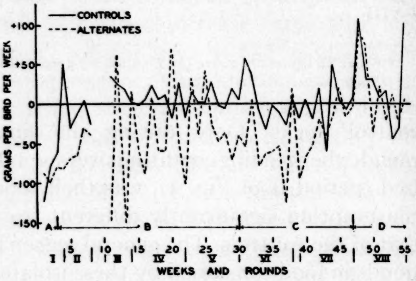

FIG. 5.—Average gain or loss in body weight, in grams per bird per week. Feeding time was restricted during periods A, B, and C to 30, 60, and 90 minutes, respectively. During D, mash was present all day, and scratch grain was fed for 60 minutes. All birds were fed two times daily.

When the graph for the alternates is scanned in a more general way, the record indicates that the alternates were fairly consistent in losing weight during the first 47 weeks of the experiment. Whether analyzed by population differences or by weekly variations, the difference in ability to maintain weight between controls and alternates during rounds III or IV to VI, inclusive, is statistically significant. During these rounds the controls gained slightly (6 or 7 gm.) and the alternates lost, on an average, 37–40 gm.

The same trend continued after the changed treatment that marked the beginning of round VII (see Table 1); but, with the presence of mash throughout the day in round VIII, the differences came to have slight probability of being statistically significant. Even here, the conservative statistical analysis by population shows that the difference of some 18 gm., for these last two rounds, considered together, is probably significant. The mean difference throughout the whole 55 weeks amounted to 36 gm. when weekly means are compared, and this has high statistical probability with a P-value of 4×10^{-6}.

In general, the weight lost during the week, or 2 weeks, of membership in the shifting flock of alternates during rounds I–VI, inclusive, was regained during the ensuing 3–6 weeks in isolation. The situation that existed in the last two rounds is more complicated, but the general indications support those from the earlier months of the experiment.

RATE OF EGG PRODUCTION

Analysis by weekly means.—The rate of egg production is summarized in Table 6 and Figure 6. Among other variables, individual differences in size of clutches of eggs and in the interval between them, even among isolated hens or members of the relatively stable control flocks, helped produce irregularity in the consecutive weekly rates of egg-laying. A five-point moving average was applied to the raw data to smooth observed variations and so reveal general trends. The resulting curves have a similar general pattern that represents the annual laying cycle and agrees, in general, with normal cycles, reported, among others, by Atwood (1928) and Winchester (1940). Details in the following summary are based on the actual, unsmoothed records.

Controls, alternates, and isolates started with practically identical laying rates in June, 1942, immediately after receipt in Chicago. They soon began to deviate from each other, but the general pattern of deviation did not become clear until the seasonal decline was well under way. The alternates reached their first off-season low point before either of the others. At that time they were housed for six weeks out of successive periods of 8 weeks in rather roomy isolation pens (p. 322) and were in the pen of alternates during the remaining 2 weeks of each round. At their lowest point the alternates ceased laying for a week. The wide variation near the low point of the laying curve for the alternates is probably an effect of the chance composition of a flock of only seven hens with a continuously changing membership. The chances are good that in a shifting flock of this size the variation in number of steady layers might be extreme.

The control flocks reached a low point of about the same level as that shown by the isolates but arrived at it some 5 weeks later. They also began the upswing decidedly later and reached the same mean high level as the isolates again some weeks later; their summer decline apparently had just begun at the end of the experiment.

The isolated hens began their new laying cycle sooner than the controls; it progressed rather steadily to an earlier peak, and the 1943 seasonal decline began earlier than the controls. The alternates also started their steady seasonal upswing of egg production before the controls. Their seasonal trend was broken just before the change in experimental treatment (p. 323) that began with round VII.

Among other factors, marked seasonal depression in laying was coincident with molting. The intensity and duration of the molt varied greatly among the individual hens. The feather record showed that there were more controls with immature feathers after the thirtieth week (round VI) than experimentals. The seasonal minimum comb size for each hen

TABLE 6

SUMMARY OF WEEKLY MEANS OF INDIVIDUAL EGG PRODUCTION

Rounds	Weeks	Eggs per Hen per Week			*P*-Values by Statistical Analysis*					
					By Populations			By Weeks		
		Controls	Alternates	Isolates	Controls versus Alternates	Controls versus Isolates	Isolates versus Alternates	Controls versus Alternates	Controls versus Isolates	Isolates versus Alternates
I–II	1– 8	2.7	2.5	2.7	....	....	....	....	....	....
IV–VI	15–38	1.4	1.1	1.9	27×10^{-3}	....	1×10^{-6}	3×10^{-2}	2×10^{-3}	1×10^{-6}
III–VI	11–38	1.4	1.2	1.9	6×10^{-2}	....	1×10^{-4}	5.6×10^{-2}	5×10^{-4}	2×10^{-4}
VII	40–47	3.4	2.3	3.6	4×10^{-2}	....	6×10^{-6}	2×10^{-4}	....	1×10^{-3}
VIII	48–55	3.6	2.3	3.1	2×10^{-2}	....	....	2×10^{-4}	5×10^{-3}	2×10^{-4}
VII–VIII	40–55	3.5	2.3	3.4	2×10^{-3}	....	2×10^{-3}	2×10^{-4}	....	4×10^{-3}
I–VIII	1–55	2.3	1.8	2.5				2×10^{-7}	4×10^{-2}	6×10^{-7}

* Dots show that the *P*-values are greater than 1×10^{-1}; they must be 5×10^{-2} or less to be taken seriously.

occurred during the molt; and the date at which the greatest comb regression was obtained may serve as a better critical measure for comparing individual molting time than does an estimation of feather maturity, although it does not indicate the duration of molting. Among the experimentals the maximum comb shrinkages were recorded between the eleventh and the twenty-sixth weeks, with the average at the eighteenth week; the range for the controls was from the twelfth to the thirtieth week, and the mean fell at the twenty-third week. This mean week for the controls came before the trough of the depression in their laying cycle (Fig. 6), and the thirtieth week approximates the initial rise in egg production for this group. Similarly, the eighteenth-week mean and the twenty-sixth-week range limit mark similar points in the cycle of the experimentals when in isolation and not further disturbed by conditions related to alternation. These observations show the expected correlation between recovery from the molt and an earlier rise in ovulation rate.

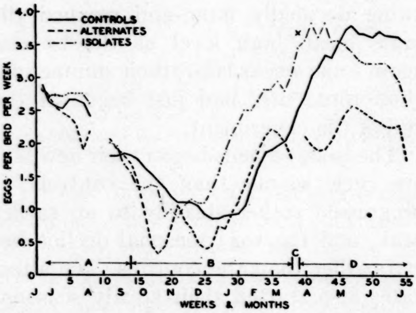

FIG. 6.—Average number of eggs laid per bird per week. During period A each alternate was in the experimental pen for 7 days, and in B and D each alternate was resident for 14 days. Alternates were rotated from isolation for 38 weeks; no changes were made in C; and in D, rotations occurred between three experimental pens. The × for the thirty-ninth week gives the average for the newly formed third flock of alternates.

During week 39 (period C), we ceased alternating hens between isolation and a single experimental pen in the chicken-house and made ready for a rotation between three pens in the chicken-house; details of this change in experimental procedure are given on page 323. The new alternates continued steadily under conditions identical with those for the controls except for rotation of the hens longest in their respective flocks. The change in trend of egg production between controls and alternates in rounds VII and VIII is striking.

The alternates laid fewer eggs than the controls; and, however analyzed, after the first rounds, the results are statistically significant. The differences in rate of egg-laying for these two sets of hens in rounds VII and VIII are the most critical data available on this point, since both controls and alternates were then housed under similar conditions. Likewise, after initial adjustment, the isolates laid more eggs than the alternates; and the differences were also usually significant. During round VIII the isolates were beginning their seasonal decline, and the average for the controls was still high. Otherwise, the consistent similarity in rate of egg production between isolates and controls is noteworthy. None of the observed differences are statistically significant when analyzed by population; but some of the trends are clearly distinct from each other when analyzed by weeks, though first the isolates and later the control hens lay at the faster rate. These departures appear to be related to the lack of coincidence in the respective laying cycles rather than to real differences in egg production for isolated and control hens.

The changes associated with transfer from isolation to the flock of alternates before round VII involved exposure to differences in temperature, in length of day, and in total illumination—at least in some isolation pens (p. 322)—as well as a change from visual and physical isolation to membership in a socially unorganized and active flock. Hence, only the last two rounds, and then only between controls and alternates, yield clean-cut experimental results, as far as egg production is concerned—results that can be attributed with certainty to the effect of alternating, as compared with steady, flock membership.

If there had been a consistent decrease in length of day for hens in the poultry-house pens, as contrasted with those in isolation, many of the observed differences in rate of egg-laying could be readily explained (Rowan, 1925; Wetham, 1933; Bissonnette, 1936; Warren and Scott, 1936). As has been shown earlier (p. 322), especially on dull winter days, the photoperiod was not consistently shorter in the flocks of controls and alternates; hence, this easy and obvious explanation of the reduced rate of reproduction among the alternates in rounds I–VI is not the full explanation.

Apparently, there was some carry-over to the flock of alternates of the tendency toward increased egg-laying that characterized isolation, since the seasonal upswing started earlier in these shifted birds than in the more socially static control flocks. During the first weeks of late-winter round VI, the alternates showed a steady trend toward laying more eggs for any given week than did the accompanying controls; the *P*-value of the difference in weekly means analyzed by weeks is 2×10^{-4}.

Whatever caused the temporarily higher rate of reproduction among the alternates appears to have lost potency during February—well before the end of round VI. The trend in egg production changed abruptly among the alternates when compared either with the isolates from which they were being regularly withdrawn or with the control flocks. Is this change a real expression of the effect of social interaction among the flock of alternates that were (*a*) pecking each other more frequently, (*b*) eating less, and (*c*) losing weight; or is it, perhaps, merely another rather extreme change in trend such as occurred in November?

Only further work can provide a definite answer.

Considering the marked difference in mean rate of egg-laying between controls and alternates during rounds VII and VIII and remembering that two-thirds of the new alternates had recently been members of two well-organized flocks, we are strongly inclined to take seriously the suggestive differences in egg-laying shown by Table 6 and Figure 6 and the tentative conclusion that continued social disturbance among the alternates was a major contributing factor toward decreased egg production throughout most of the experiment. Support for this interpretation will be given immediately.

TABLE 7

THE MEAN NUMBER OF EGGS LAID PER BIRD PER WEEK IN ISOLATION AND IN THE EXPERIMENTAL PEN, AS COMPARED WITH ACCOMPANYING CONTROLS*

Rounds and Dates	Alternates		Isolates					
	Week 1	Week 2	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
			P: 0.045					
I–III (6/17–9/25)	1.9	.....	1.7	2.9	2.6			
			P: 0.000004					
				P: 0.0001				
IV–VI (9/26–3/12)	1.4	0.9	1.1	1.9	2.3	2.2	2.0	2.0
	P: 0.0005		*P*: 0.0005					
	Controls							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
I–III	2.7	2.3	2.4	2.0	.....	.....	.....	.....
IV–VI	1.1	1.3	1.1	1.1	1.2	1.3	1.3	1.0

* For conditions see pp. 321 and 333.

Changes in rate of egg-laying during critical weeks.—Because we did not have sufficient room, our isolated hens were not housed under physical conditions identical with those under which the control and experimental flocks lived. We have used a number of different analyses in an attempt to repair this basic experimental weakness.

The alternates and isolates were composed of the same individuals during the first six rounds. Each individual experienced successive transfers from isolation to membership in the flock of alternates. The impact of repeated exposure to the different physical and social conditions associated with these changes is recorded in Table 7 in terms of changes in the rate of egg-laying. Each figure represents the average performance of all hens during rounds I–III and IV–VI. Data for incomplete weeks in isolation or in the experimental pen were omitted; otherwise, as usual, there was no selection.

During the first three rounds, when each hen remained in the flock of alternates for 1 week only and then was moved back to isolation, the lowered rate of egg production developed while in the changed physical conditions and among the socially active flock; it persisted during the first week of isolation and then showed recovery to a new level. The recovery between the first and second week in isolation gave an average increase of 1.2 eggs per hen and has the excellent statistical probability indicated by a *P*-value of 4×10^{-6}. The rate of egg production apparently approached an equilibrium at this level. The drop in egg-laying from the last week of isolation to the week spent among the alternates can be taken fairly seriously, since the mean difference has a *P*-value of 0.045.

In the winter rounds, IV–VI, the indicated recovery from a mean of 1.1 eggs laid in the first week of isolation to 1.9 in the second has good statistical probability of being significant (*P*-value, 5×10^{-4}). Equilibrium in rate of production came at a somewhat higher level than was reached during the second week of isolation; hence, full recovery from 2 weeks spent in the pen did not come until after 2 weeks of isolation. The drop in the mean rate of egg-laying between the last full week of isolation and the first week as an alternate also has good statistical probability (*P*-value, 1×10^{-4}). There was a further drop from a mean of 1.4 to one of 0.9 eggs laid during the second week of flock membership, and the statistical probability is also high (*P*-value, 5×10^{-4}).

The control hens, members of fairly stable social flocks and housed under relatively constant conditions, showed no regular variation with the passing weeks in egg-laying except those associated with the marked seasonal trends. The numbers of eggs laid by controls in odd-numbered weeks did not differ significantly from the numbers laid in even-numbered weeks of the experiment. There was no real difference between number of eggs laid during alternate weeks by the experimental hens in rounds VII–VIII when they were being circulated among three flocks.

What might have been a crucial test of the effect of a change from a relatively stable social situation to an unstable one, uncomplicated by uncontrolled shifts in temperature and light, came with the transfer of hens from control flocks G and P to form two of the experimental flocks at the start of round VII. The significance of the test was partially hidden by unexplained variations in rate of egg-laying, which will be described in detail in the following paragraph. The hens of both these flocks were kept throughout in henhouse pens, and so only their social situation was changed (p. 323). All the hens were in the midst of their seasonal upswing in egg production. The weekly averages per hen for these two flocks and for flock W, which had been and remained a control flock, are shown in Table 8.

The three control flocks were laying eggs at essentially the same rate during the closing weeks of round VI. For some reason unknown to us, during the interval between rounds VI and VII, when no birds were interchanged and when these flocks were under the routine to which they were accustomed, hens in flocks G and P, considered together, decreased their egg production to the unseasonably low level of 1.5 eggs per hen and immediately returned to their former rate of egg-laying in the following week. Meantime, those in flock W showed an unusually high production in the thirty-ninth week (3.4 eggs); the difference has a *P*-value of 0.022. This *P*-value is low enough to be taken seriously statistically, even though we do not understand its biological significance. It represents one of the vagaries that comes from working intensively with small numbers.

If the aberrant results for week 39 are disregarded, we find that W continued in its seasonal upward trend in egg-laying, with the increase between week 38 and weeks 42-43, combined, approaching statistical significance (*P*-value, 0.053); the increase between the last 5 weeks of round VI and the first 5 weeks of round VII is 1.3 eggs and has good probability of being statistically significant (*P*-value, 0.01) when analyzed by compared populations. In contrast, flocks G and P, when undergoing rotation of membership, tended to level off at the egg-laying rate reached while they were still controls. None of the changes in later weeks with these two flocks are statistically significant when compared with week 38 or with performance during the closing weeks of round VI.

When the mean rates of egg-laying are compared week by week for the 8 weeks of round VII, it may be seen that hens of flock W laid, on the average, 1.2 eggs per hen per day more than did those in the former control flocks, G and P; and the difference has excellent statistical probability of being significant (*P*-value, 0.0006).

The seven experimentals selected to form the third of the new rotating flocks included four hens taken from isolation at the beginning of the thirty-ninth week and three hens that had just been members of the alternating flock. They laid an average of 3.7 eggs during week 39, a statistically insignificant increase over their performance the preceding week. Their production then fell off and reached a low of 1.1 eggs per hen for week 41 (*P*-value of the difference between weeks 38 and 41 is 0.01), and fell slightly lower the following week before coming up to a level approximately that of the accompanying experimental flocks G and P. The other changes in this flock recorded in Table 8 are not statistically significant.

It follows that the present analysis supports previously discussed indications that birds in socially unstable flocks laid fewer eggs than did similar hens in flocks with stable organization. It is also apparent that we were not in full experimental control of the factors that determine the rate of egg production.

TABLE 8

THE MEAN NUMBER OF EGGS LAID PER BIRD PER WEEK BEFORE AND AFTER THE CHANGE IN METHOD OF ROTATION (P. 323)

Round	Week		Mean Eggs per Week G Plus *P*		W		Selected Experimentals	
VI	34		2.4		1.7			
	35		1.8		0.9			
	36		1.9		2.4	↓		
	37		2.0		2.0	*P*: 0.01		
	38		2.2		2.4		3.0	
		P: 0.16						*P*: 0.01 ↗
No change	39		1.5	*P*: 0.022	3.4		3.7	
		P: 0.018				←*P*: 0.053		
VII	40		2.2		2.7		2.4	
	41		2.6		3.5		1.1	
	42		1.7		2.7		1.0	
	43		1.7		3.8		2.5	
	44		2.2	*P*: 0.0006	3.1		2.0	
	45		2.1		3.7		2.0	
	46		2.5		3.8		2.8	
	47		2.7		4.1		3.1	

CHANGES IN SIZE OF COMB

Combs of hens of the same breed tend to become smaller with decrease in ovarian activity because of a decrease in the production of male hormone by these hormonally hermaphroditic animals (Allee, Collias, and Lutherman, 1939, and citations). Combs become reduced in size in hens that are ill, molting, or on sufficiently short rations. Conversely, the comb tends to become larger with decreased lighting (Domm, 1930).

Measurements of comb size were made by finding the length of the blade and the height of the spike most nearly over the eye (the same spike was used throughout for each individual). Each comb was measured weekly and, in addition, the experimental hens were measured when transferred to and from isolation. Measurements from heavily molting hens were excluded, as were all data concerning comb size from rounds III and IV, because of the relatively heavy and general molt at that time.

As shown in Table 2, the hens, when received, all belonged to one population, so far as could be determined by variations in size of comb. Our hens showed the most rapid increase in size of comb when laying was resumed after the autumnal molt. Since molting and laying cycles were not synchronized among controls, isolates, and alternates, comparisons for a given week, or set of weeks, are much more complex than is indicated by the simple, direct comparisons used here.

In each of the six rounds not too severely affected by molting, the change in size of comb was such as to yield a relatively smaller comb among the alternates, as compared with the controls. This tendency has convincing statistical probability only in rounds VI and VII at the time of the strong seasonal upswing in egg-laying (*P*-values, 6×10^{-5} and 1×10^{-7}, respectively). When all six sets of comparisons are considered, the relative shift toward a smaller comb in the alternates averaged 6.75 mm.; this difference is interesting statistically, since it has a *P*-value of 0.043. In three of the four rounds (I, V, and VI) when the same hens were alternated between isolation and membership in a socially unorganized flock, the isolates showed a significant tendency toward having a larger comb—a tendency that runs counter to expectation if (*a*) the size of comb in these birds were being determined by light intensity and if (*b*) the light were actually stronger in the altered greenhouse, where the hens were isolated, than in the poultry-house, where they were housed as alternates. The same point can also be made in a different way. As with many other comparisons given earlier in the present report, despite differences in housing, the changes in size of comb of controls and isolates were essentially similar when mean changes are considered; the *P*-value of the observed differences is 0.4.

Again it is profitable to examine carefully the data regarding comb size just before and following the inauguration of pen-to-pen alternation at the start of round VII. The combs of the continuing control flock W became slightly larger during the 3 weeks immediately before and after the beginning of week 40 (0.34 mm., length plus height), but the change was not statistically significant (*P*-value, 0.16). In the same interval the combs of the former control flocks G and P decreased 2.1 mm., on the average; and this decrease may be taken seriously (*P*-value, 0.01).

It is also possible to compare the comb changes of flock W with those of G and P before and after the latter became members of experimentally disrupted flocks. During weeks 36–39, comb growth in flocks G and P compared favorably with that of their fellow control flock, W; in fact, the combs of the former showed, if anything, the greater growth. For the 8 weeks of round VII (weeks 40–47, inclusive) combs of the members of flocks G and P, now subject to social disturbance, decreased 5.4 mm., on the average; and during the same period the combs of the continuing controls W increased 8.4 mm. The mean difference of 13.8 mm. has a *P*-value of 0.042, calculated by the population method. A trend is indicated —an indication that is supported by other comparisons, including some that are not summarized here.

These changes in comb size seem to come as an end result of the different social conditions that obtained in flock W, as compared with flocks G and P during round VII. The hens had all been housed for months in neighboring pens, and they continued to live under similar conditions of lighting, temperature, space, and food. The only known difference is that the hens of flocks G and P were now subjected to recurring social disruptions.

In general, the changes in length plus height of comb support the evidence from the trends in egg production, although not always in a clear-cut fashion. Together, these two lines of evidence indicate that there was a change in the functioning of the reproductive system associated with a disturbed, as contrasted with a steady, social state.

SENIORITY AND SOCIAL POSITION

During the first six rounds (38 weeks) of our experiment each of the seven hens in the shifting flock of alternates progressed steadily from being the newcomer for a day (rounds I–III) or for 2 days (rounds VI–VI) to a similar brief period of seniority just before being removed for another extended period of isolation. Normally, each newcomer entered the flock in the omega social rank, typically showed a steady advance in rank with seniority in the flock, and emerged as an alpha hen, socially speaking, during her last days before again being isolated. This relation between pen seniority and social status is illustrated in Figure 7. The correlation coefficient (*r*) between days in the experimental pen and social rank is 0.60 ± 0.02. Exceptions occurred that kept the relationship from being diagrammatic. These were based primarily on individual differences between the hens. Extremely aggressive hens introduced into a flock that contained several nonaggressive ones might gain high, or even alpha, social rank before they attained flock seniority; and, conversely, unaggressive hens might never reach the top of the shifting peck-order. The lines in Figures 7 and 8 show the regression coefficient as located by the method of least squares. In order to avoid a negative correlation coefficient, rank 7 was rated as unity (1), and each half-rank was counted as a full step up in

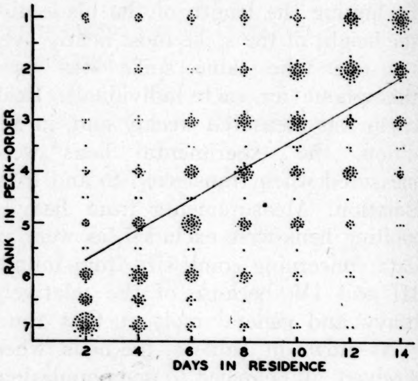

FIG. 7.—Correlation between seniority (abscissas) and social rank (ordinates) in the pen of alternates during the first six rounds. The half-steps in rank come from membership in social triangles.

the peck-order. According to this system, alpha rank carried a value of 13.

The frequency of pecking among the alternates varied according to the mixture of aggressiveness and submissiveness of the hens present at the given time. Pecking was likely to be intense when an inherently aggressive hen found herself with low social status in a strange flock. The frequency and severity of pecking might fall decidedly when the senior hens were essentially mild individuals and were only aggressive enough to attain and hold the rank to which they were entitled by their seniority. They make so-called "benign despots."

With the change in procedure at the start of round VII (pp. 323, 332) there was a change in the correlation between pen seniority and social rank. The same general rule still held but with modifications, produced perhaps, in part, by individual acquaintanceships carried over from flock to flock and by the fact that the hens were now being rotated between pens that were physically similar rather than being moved from isolation to a strange physical and to a complicated social environment. In order to reduce the frequency with which each hen would meet former flockmates within the time that they can apparently remember other hens as individuals, the order of rotation was changed every 2 weeks; and if, for example, one hen from pen 1 was placed in pen 3, the next to be taken from pen 1 would be placed in pen 4. Hens from the other two experimental pens were similarly distributed alternately as the fortnightly residence of each was concluded.

Despite such care, three or more pairs of acquainted hens met during each biweekly rotation. Recognition was evident in a number of cases; but previous reaction patterns were altered at times, since the hens met in new social combinations with different positions in the new peck-order. Thus, when the formerly inferior GG met her erstwhile superior GW after only 4 days of separation, GG typically gave an avoiding reaction. After 12 days of membership in this same flock, GG attacked her newly introduced, former superior GY. The social development of GG under new conditions was affected by her past and still-remembered pair relationships and by her growing seniority in the flock, as well as by the internal factors that make for social success among hens (Collias, 1943).

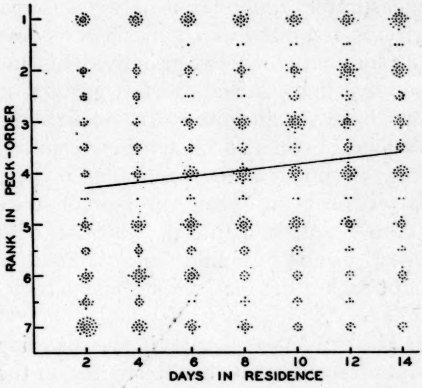

FIG. 8.—Correlation between seniority (abscissas) and social rank (ordinates) during the last two rounds.

Under these more complicated conditions the correlation coefficient between pen seniority and social rank dropped to $r = 0.24 \pm 0.03$, a value that is still statistically significant. Apparently, seniority, taken alone, is more important when each newcomer moves from isolation into membership in a flock in which she must win her way entirely by present personal performance than when she comes from another flock with certain holdover acquaintanceships.

DISCUSSION

The present experiments were primarily concerned with measurable differences between socially stable control flocks of hens, organized according to the peck-right system common among them, and flocks that were in a continual state of social reorganization. The procedures also allowed comparisons (*a*) between the performance of hens during an extended period of isolation and the activities of the same individuals when among the actively fighting flock of alternates and (*b*) between isolates and the members of the control flocks.

The problem is related to, though distinct from, that which centers about the measurable differences between social groups organized on the basis of a series of pair contacts between individuals and totally unorganized social groups in which no social dominance, no fighting, and no obvious social tensions exist. It may be possible to experiment on this latter problem, using groups of mice trained not to fight, in contrast with other groups of similar mice trained to fight each other, and so establish a social order.

The primary emphasis in the planning and prosecution of these tests was on the relative performance of hens in organized and unorganized flocks; and in the discussion, as in the foregoing pages, we shall avoid interesting leads except as we are forced to consider them in connection with the basic problem. Some of the matters that are being brushed aside include (*a*) environmental controls of the annual egg-laying cycle; (*b*) an analysis of the role of individuality in social organization and in the performance of socially organized flocks; and (*c*) a consideration of some fundamentals of the peck-order relationship among hens that are not directly related to our primary problem.

The evidence at hand strongly indicates that these intraflock-bred White Leghorns all belonged to one common population when received and that their subdivision into controls, as contrasted to alternates and isolates, was a random separation. The differences in performance that appeared during the progress of the experiments were, therefore, a result of the respective treatments or of chance variations rather than of being an expression of inherent differences between experimental and control populations.

Members of our stably organized flocks pecked each other less, obtained more food, and maintained body weight better than did hens that were undergoing constant social reorganization. The isolated hens could not see their fellows, much less peck them. In other possible comparisons—namely, food consumption, maintenance of weight after recovery from that lost while alternates, egg-laying, and changes in size of comb—performance of the isolates resembled that found in the control flocks rather than their own individual performances when they were members of the flock of actively pecking alternates. It is important to note that the isolated hens laid as many eggs as did those in the socially stable flocks, despite the handicap of recurring weeks of membership in the inefficient, socially unstable flock of alternates. The indications are that, other things being equal, hens in complete isolation would produce more eggs than would those in flocks, even when the latter were well organized.

Early in the experiments, after rotation was established, the senior hen, in length of time among the alternates, was removed each day and replaced by the hen longest in isolation. During the three rounds when daily alternations were practiced, evidence accumulated that 1 week was insufficient, at least with some hens, completely to eliminate the effect of strangeness of the physical surroundings furnished by the larger pen when combined with the strangeness of being a member of a flock after a period of isolation.

During these first weeks the intensity of pecking was less than is usual among White Leghorn hens when a stranger is introduced into a flock that is well adjusted to group life in their particular pen. Pecking activity became more intense when, beginning with the fourth round, the rotation schedule was changed to allow residence of 2 weeks in the pen of alternates. The number of pecks delivered among the alternates rose from a mean of 177 per hen per week for rounds I–III to one of 209 for rounds IV–VI, inclusive. Meantime, with increased stability of social organization, the rate of pecking decreased decidedly among the control flocks (Table 3).

The effect of strangeness of the whole environment (biotic and physical), the relative speed of rotation, and the sudden change from a commercial flock to laboratory pens may well have affected the lack of significant statistical differences between the amount of pecking of controls and that of alternates during the first three rounds.

When alternations were made from pen to pen, rather than between pen and isolation (rounds VII and VIII), strangeness of physical conditions must have been largely eliminated. To the human eye, at least, one pen was very much like another. Under these conditions there was some evidence that the birds, when moved in steady succession between the pens, displayed learned social reactions that caused them to fit into new social levels with less time for adjustment than when birds were removed from extended isolation and placed in the pen of alternates. They were steadily in about the same physical and biotic environment, except for the regular change in individual members of the flock; and apparently the hens became somewhat adjusted to being rotated every fortnight, even though they never ceased to react to strangers in typical hen fashion. The conditioning that resulted from being shifted about in a similar environment may be reflected in the decreased difference in change of body weight between controls and alternates in these last two rounds. That is not the whole story, since during the last round the hens generally had better opportunity to feed from the mash that was constantly present during the day than they had had during previous rounds, and with food more available the hens did less pecking than at any previous time in these experiments.

The effects of this same conditioning are shown by the change in social progression with seniority (p. 339) when the hens were rotated among three unorganized flocks, as compared with that shown when they were transferred to an unorganized flock from isolation. The possible effect of acquaintanceship with some of the other individuals in the constantly rotated flocks is also a part of this decreased correlation of seniority with social status.

The establishment of a peck order in a flock of hens involves a series of learned relations among flockmates that includes learned toleration as well as learning which individuals to avoid and which are socially inferior. The rapidity with which hens adjust themselves in a flock and come to exhibit fitting behavior to each of their different flockmates speaks well for their adaptability when confronted with this particular set of problems, but they have slight power of retention.

Our data suggest, as Tinbergen (1939) and Guhl (1942) have previously concluded, that individual hens develop special habits in relation to every other individual in their flock, providing the flock is not too large. The combined effect of learned dominance, toleration, and avoidance is indicated by the decrease in pecking frequency among the controls with increasing acquaintance between individuals.

Such learned and remembered relationships form the basis of the social inertia that reinforces, and at times somewhat replaces, aggressive behavior patterns in flock organization (Allee, Collias, and Lutherman, 1939). Many phases of learning as a factor of integration were largely eliminated among the alternates, particularly during the early rounds of the present experiments; and the resulting differences make one of the basic contrasts between them and the control flocks.

Some of the observed deviations from expectation are explicable on the basis of known variations in individuals. Thus, WY stood next to top in the peck-order in control flock W. At times she became very active in pecking her subordinates and would even attack her superior. As a result, flock W behaved in some respects more like the alternates than did either of the associated control flocks. WY had one of her erratic bursts of pecking during round VII and so contributed strongly to the atypical rise in number of pecks delivered by the control flock in that round (Fig. 3). It is well to remember that such aberration may exist in otherwise well-organized, stable flocks, for it will help to avoid the temptation to make too sweeping generalizations about differences between such flocks and those that are in the process of social organization. Such an aberrant individual temporarily escapes from the routine of learned patterns of social behavior, and the reactions of the whole flock are affected. The rapid decline in number of pecks in flock W during round VIII depended somewhat on the cessation of erratic behavior by WY. The decline was affected still more by the better opportunities for feeding in round VIII and by learned avoidance on the part of certain continually submissive hens. Shortage of food increases the close competition between pairs both in organized and in unorganized flocks.

The decrease in intraflock fighting among members of an organized group with an established peck-order, the greater consumption of food, and the resulting better maintenance of weight all have immediate survival value for members of such flocks. To be effective, factors on which selection can work must be related to reproduction. As mentioned later (p. 344), there is now good evidence, other things being equal, that dominant males frequently have a better opportunity to leave more numerous offspring than do those low in social rank (Scott, 1942; Guhl, 1941, 1942; Collias, 1944).

On the female side, Schjelderup-Ebbe (1935) believes that the female must not be too dominant in relation to males if successful breeding is to result, and Noble and Wurm (1944) apparently come to a similar conclusion. This is not our present problem. According to Sanctuary (1932), there is no marked difference in egg production by different individuals when all hens in the flock are relative strangers; but, when strange hens are introduced into a flock of acquainted hens, the newcomers show a reduced rate of egg-laying and may even cease to lay for the time being. Sanctuary also has evidence that hens low in the social order may not realize their full potentiality in egg production.

In our pens the relation between frequency of pecking in organized flocks showed an inverse relationship (*a*) to food consumption and (*b*) to maintenance of body weight, and also (*c*) to rate of egg-laying as well. Even in round VIII, when mash was available all day and there was no real difference in maintenance of weight between controls and alternates, still there is good probability that the alternates laid significantly fewer eggs than did the controls (Table 6). This might be expected on general grounds. If this suggestion can be taken at its face value, all our findings become consistent and indicate the existence of survival values associated with flock organization that may, if they exist in nature, give a direct basis for natural selection of socially organized flocks.

The complication furnished by differences in the housing of the isolates and the penned flocks has already been discussed at some length (p. 323). To conclude that all the changes in rate of laying and in size of comb result from physical differences in the two situations gives much more difficulty than does the assumption that, to a considerable extent, they resulted from known differences in social activity.

The effects of intraflock pecking on rate of egg-laying and on maintenance of weight are matters of considerable general interest. The social organization of flocks of hens is based on a series of dominance-subordination interactions of bird against bird that depends ultimately on individual aggressiveness or the lack of it. It would follow that flock organization which results from individual competition (together with learned toleration) gives a co-ordinated social unit that is better fitted to survive than the same number of grouped animals would be when they are fighting among themselves (Allee, 1942; Collias, 1944). Competition at the individual level here helps give rise to an organized group better fitted to co-operate as a social flock.

Application to conditions in nature must be made with caution. Our experiments were on the well-domesticated common hen and were carried on under artificial laboratory conditions. There are some indications that the suggested transfer of information to life in nature is possible, at least for certain groups. All mammals that live in organized groups, like the baboon hordes (Allee, 1931), illustrate a part of the same general set of principles. In these hordes there is leadership, as well as intragroup organization, on the basis of dominance. Flocks of hens are said to show little connection between high position in the peck-order and leadership (Fischel, 1927) and hence are essentially simpler social groups than are those of the baboons.

Jenkins (1944) reports that a family of four blue geese (male, female, and two young of the year) "showed organized despotism through its strong integration and co-operation, resulting in the dominance of the family" as a unit among the mixed winter population of geese and ducks in the Jackson Park bird sanctuary in Chicago. No pecking was observed within the family until the new breeding season started; the adult male then pecked the young birds, and only the mated couple was left as a social unit. This family did not appear to be organized as a result of individual aggression; hence this is not a complete field illustration of the point in hand.

Some phases of our work seem to be connected with field studies on the bob-white (*Colinus*) and the valley quail of California (*Lophortyx*). Errington (1941) says of the former: "The statement by Stoddard (1931:170) that Bob-white coveys tend to keep their organization of normal size through repeated combinations is emphasized by data on the possible role of 'tradition' in determining toleration limits in wintering territories. . . ." (p. 98). "The likeliest mechanism behind these toleration phenomena seems to be dominance by veteran individuals that have their own ideas as to what constitutes desirable or safe numbers of birds in specific habitats" (p. 99).

These statements are in accord with observations that there are "outcasts" from bobwhite coveys and that these "outcasts" have low social status or are rejected when they attempt to join another covey. Such relationships are important for the problem at hand, since Gerstell (1939), found that members of the grouped covey are better able to withstand low temperatures than are isolated birds. Again this case only partially carries our laboratory observations over into conditions found in nature.

The valley quail of California has a social order within the covey (Emlen, 1939; Emlen and Lorenz, 1942). Emlen reports (*vide* Errington, 1941) that there is "very little evidence of policing or of peck order in coveys until in February when .... older birds generally rank highest," except when the older ones have recently come from other coveys and are therefore newcomers and relative strangers. When the general population is low and when apparently the members within the covey are well acquainted with each other, wandering strangers find it difficult to gain admission. Conversely, when the population is large and wanderers are numerous, they are better able to secure acceptance by a strange covey. These observations on *Colinus* and *Lophortyx*, taken together, come somewhat more close to providing a transition from our laboratory flocks to field conditions. Errington[4] comments on this general point as follows:

> In my opinion your data on white leghorns held continuously in the process of flock organization should most nearly reflect conditions found in the field during periods of critical over-population. With the animals I have studied, a high degree of social instability is not only a constant invitation to predators but also .... [is accompanied] by irregularity in breeding and other complications.

The closest connection we know is made by published and some unpublished observations of Professor J. W. Scott.[4] Scott (1942) reported at length concerning the social organization of sage grouse males on their breeding grounds. Various social ranks can be recognized: (1) "master-cocks," which do most of the copulating; (2) "subcocks," which are less successful sexually; (3) "guard cocks," which are still less active in mating; and (4) the remaining males, which are widely distributed, singly or in pairs. Scott now has told one of us that 3 years of continued observations on the mating of the sage grouse show that his original account was substantially correct but that there is a considerable amount of flexibility.

The cocks shift away from a spot covered with snow and move back to the accustomed position when the snow melts. In one instance this shifting-away from a snow-covered mating place brought a regrouping of cocks relatively close together, so much so that there was a great deal of fighting between the closely crowded and contesting cocks. The females moving into the mating grounds with the first dawn congregated in large numbers about the area where much fighting was in progress. Later in the morning, one cock would fight the master-cock even while he was engaged in the mating process. About this time, as the light was growing stronger, some seventy-five hens moved off to a mating place where all was well organized and quiet.

This is almost a diagrammatic demonstration of the greater effectiveness of the relative quiet of the organized mating ground, as contrasted with one that is disorganized or, perhaps, in the process of reorganization. It also provides an interesting variation from the usual account of sexual selection, just as the mating relations on one of the organized mating spots illustrate the more typical interpretation of sexual selection, with most of the matings going to the dominant cock. Flocks with such organized behavior could readily have high selective values somewhat similar to those possible among the socially stable flocks of our common hens.

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[4] Personal communications in 1944, published here with proper consent.

The presence or absence of a working organization both in the mating flocks of sage grouse and in our pens of White Leghorn hens may well be of secondary importance in producing the observed differences between organized flocks and those in the process of organization. There is a good chance that the decrease in fighting and associated disturbances in the organized flocks is the basic difference. If this is, indeed, the primary influence, the sequence of events is interesting and instructive. Flocks of hens attain the peck-order type of social hierarchies as a result of fighting or threatening or by passive acceptance of subordinate status. The flock-survival values shown by organized groups result from eventual decrease in the amount of fights, threats, and obvious avoidances. According to this reasoning, any other influences that similarly decrease these more extreme exhibitions of social tension should have similar survival values; and flocks in which intraflock fighting is entirely absent and that lack peck-order organization should show to a still higher degree survival values such as we have been measuring. Experimental analysis of this enlarged problem is apparently possible with other animals if not with birds.

No cocks were present in our flocks, and hence all eggs were infertile. The simpler conditions furnished by homosexual flocks of domestic hens now having been described, we have a basis for comparison with the more complicated situation when males are present. A repetition of our experiments with flocks containing both cocks and hens should provide an interesting and probably a profitable extension of this experiment in general and comparative sociology.

SUMMARY

These experiments were performed in order to compare measurable differences between socially organized flocks of common hens, and similar flocks under constant reorganization.

1. The hens belonged to a single population before the first random separation into subgroups.

2. After an initial period of adjustment the alternating hens in the flocks that were steadily undergoing reorganization pecked each other more than did members of the organized control flocks.

3. They also consumed less food.

4. Individuals in control flocks ate at about the same rate as did isolated hens, although the latter had access to food for longer periods each day.

5. Hens lost weight while members of the unsettled flock of alternates; controls and isolates tended to gain weight.

6. There are strong indications that the hens in the socially unstable alternating flocks laid fewer eggs than they did when isolated or than their fellows did as members of the socially organized control flocks.

7. Hens tended toward smaller comb size as alternates; their combs increased in size as isolates. The control hens closely resembled the isolates in size of comb.

8. There was a significant positive

correlation between seniority in the alternating flock and social status, which became less but still statistically significant when hens were rotated from flock to flock (rounds VII–VIII).

9. These differences suggest that organized flocks of hens possess qualities that might have survival values in nature.

10. There is some justification for thinking that these laboratory findings are indicative of similar relations in the wild. If so, individual-against-individual competition, such as brings about the peck-order type of social organization of flocks of hens, may serve to build a co-operative social unit better fitted to compete or to co-operate with other flocks at the group level than are socially unorganized groups.

11. The indications are that social organization in these flocks is of value not as an end in itself but because it tends to reduce fighting and other extremes of social tension.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

THE VERTEBRATE EYE AND ITS ADAPTIVE RADIATION,[1]
BY GORDON L. WALLS

Reviewed by GEORGE WALD
Harvard University

IN THIS beautiful book Dr. Walls devotes an extraordinary range of information and insight concerning the eye and vision, and a great gift for clear and informal exposition, to the attempt to say too much too confidently to too varied an audience. In it a great store of solid information, sound reasoning, and shrewd judgment are mixed inextricably with Dr. Walls's very distinctive predilections, doubts, prejudices, and intuitions; and the whole is qualified by concessions in form and substance designed to hold the attention of the lay reader. The book represents, in fact, a *katharsis* such as few persons have achieved in their scientific writings.

The volume is divided into three sections: Part I, "Basic" (139 pages), is an introduction to optics and the general anatomy, embryology, and phylogeny of the eye, ending with a short synopsis of the whole of vertebrate phylogeny. Part II, "Ecologic" (408 pages), comprises the bulk of the book and discusses with great ingenuity and circumspection the adaptations of eyes to the varied conditions of vertebrate existence. In this section the author has principally developed the leitmotiv of the book as a whole: "Everything in the vertebrate eye means something." Part III, "Synoptic" (134 pages), expertly reviews the anatomy of the eye within the various groups of vertebrates. At the head of each chapter in this section a very useful index guides the reader to other portions of the book in which the group is discussed. The illustrations, of which there are almost two hundred, are well conceived and superbly done. There follow twenty-three pages of bibliography and sixty-four pages of a very complete combined index and glossary. A check list of active workers in the field is also provided (p. 718), of whom one is told without elaboration, "Not all of these investigators are wholly 'trustworthy'—two or three are decidedly not, but are included here because they are too prolific to be ignored." One can scarcely see one's name on such a list without wishing that he were either more trustworthy or less prolific. The author of a seven-hundred-page monograph on the eye might feel downright uncomfortable; but Dr. Walls has modestly omitted his own name from the list.

The book contains the substance of a fine monograph on the anatomy, ecology, and evolution of the eye. These are fields to which Dr. Walls has made distinguished contributions and in which he speaks with some authority. He has performed a notable service in bringing this material together.

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[1] Bloomfield, Mich.: Cranbrook Institute of Science, 1942. Pp. xiv+785. $6.50.

This solid core of scholarship, however, is diluted and adulterated in the effort to fulfil the strange task which Dr. Walls has set himself. In his Preface he describes the book as an attempt "to interpret comparative ocular biology as a whole to those who want to know what the eye is all about, but are repelled by the pedantic terminology of anatomy texts, the mathematics of physiological optics, the scatteredness of the ecological literature, and the German language." Elsewhere he notes that "it has been written in the style of a text-book, though for a course which has yet to be given in any American university." And again: "The book should .... be entirely clear to any college student or graduate, and to any amateur naturalist—'trained' or not." Most of us know such courses, in which often a very gifted teacher—which Dr. Walls certainly is—struggles with an audience ranging from raw novices to specialists, determined not to disappoint the latter while not overburdening the former. Something, of course, must go by the board, so

> the book is not documented, *i.e.* loaded up with specific citations for every point of fact and reasoning which has originated outside of my own studies. The average reader will not miss them; and the earnest student who reads the book, and is led thereby to want to do research in its field, will have to devour all of the required reading listed in the bibliography anyway. He—and the established investigator in the field—will readily know which of my pronouncements to blame upon me alone. If not, he is free to write to me.....

This is not the book's only concession. It is written with an assertiveness which may reassure the untrained naturalist but is bound to bother others. In opening his discussion of the rods and cones, Dr. Walls states:

> Of the two types, *there can be no doubt* that the cone is the older and more primitive..... *It is certain*, for instance, that the ancestral cell lacked any means of analyzing colors. *It is equally certain* that the common ancestor of present-day rods and cones lacked any such ingenious sensitizing substance as rhodopsin..... With a slender, pointed, stimulable organelle, the outer segment, derived from a formerly vibratile flagellum .... and connecting directly to a simple afferent neuron, the pro-vertebrate visual cell *could not but have been* a high-threshold receptor .... [p. 53; my italics].

This is a strong line to take with fragile portions of one's remote ancestors. I know of no unequivocal evidence here or elsewhere in the book to support these assertions; indeed, it is difficult to imagine any. In stating these things as certain, Dr. Walls can mean no more than that they seem reasonable to him. It may be noted in passing that they do not seem equally reasonable to me.

This passage continues:

> Rods came later, as a means of extending the period of activity over a greater portion of the twenty-four hours. They were derived quite simply from cones by the enlargement of the outer segment and by an increase in the number of visual cells connected to each nerve cell. It was not desirable for all of the visual cells to make these changes..... The needs of the animal .... determined the proportion of small un-summated and larger, summated visual cells which would give him optimal visual capacity.

It should not be supposed that Dr. Walls subscribes to the peculiar view of the evolutionary process depicted here and elsewhere throughout the book. He says in his Preface:

> The reader will also soon note that my mode of expression is strongly tainted with teleology. I do not expect this to mislead anyone—it is merely an economy device.

In my opinion this book is too much a monograph to make a consistent elementary text and too much an elementary presentation to make a proper monograph and altogether too unrestrained to be either of these things. Yet, for those who can edit it, this is one of the most useful and provocative works on the eye that has ever been written. May we hope that Dr. Walls will edit a revised edition?

AN OUTLINE OF GENERAL PHYSIOLOGY,[1] BY L. V. HEILBRUNN

Reviewed by C. Ladd Prosser
University of Chicago

The first edition of Heilbrunn's *Outline of General Physiology* did much to increase interest in general physiology in America; this second edition, appearing six and a half years later, should do more. The second edition marks a distinct improvement over the first, particularly in its easier style, its more complete coverage of the field, and its omission of some of the sarcasm found in the first edition. In that edition the reader very often becomes interested in following one line of thought, only to find it suddenly terminated with a literature reference. The new edition has fewer of these abrupt conclusions by citation.

One reason for the success of Heilbrunn's *Outline* is that it is readable for biologists. In fact, it is written more for general biologists than for general physiologists. It lacks the long chapters of physical chemistry found in many general physiology texts. The approach is not so quantitative as many general physiologists desire. In treating nearly every topic, biological rather than physical data are discussed. The traditional general physiologist is amazed to find a discussion of osmosis with only a passing reference to the gas laws and to read about bioelectric potentials without mention of the Nernst equation. Surface tension is rightly presented as of little importance in properties of cell surfaces, but adsorption phenomena deserve more attention than they are given.

Besides being readable for biologists, this book is outstanding in its wealth of literature references selected from Professor Heilbrunn's very extensive reading. Reviews are cited wherever possible, and the German literature is particularly well discussed. Students might sometimes profit more, however, from a modern paper than from an older one, even though the older one might have priority.

General physiology is, as Heilbrunn points out, essentially cell and tissue physiology, and it emphasizes those functional characters which are common to all living things. But physiology must have a human application, and, unfortunately, most undergraduate courses which are labeled "General Physiology" are really a very dilute medical physiology with cursory references to cellular physiology. Heilbrunn has included a little medical material with discretion and presented it from the "general" viewpoint. For example, in the expanded chapter on vitamins the requirements of plants and lower animals are included with those of mammals, and the cellular actions are mentioned.

The chapters on enzymes, particularly the one on respiratory enzymes, have been expanded. The enzymes known in cellular oxidation are increasing rapidly, but one wishes that a chart were presented to show the parallel and series pathways by which a cell can get energy. Heilbrunn correctly states (p. 202) that coenzyme I contains nicotinamide, adenine, a pentose, and phosphoric acid, but on page 275 he omits the adenine. A little later he speaks of lactic acid dehydrogenase as being diphospho-pyridine-nucleotide (coenzyme I). This would confuse a student, especially since it is now usual to mean by the dehydrogenases the specific proteins rather than the coenzymes.

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[1] 2d ed. Philadelphia, Pa.: W. B. Saunders Co., 1943. Pp. xii+748. $6.00.

There are so many unknowns in physiology that every general physiologist has his own *deus ex machina* on which he calls at times. For some this is the invisible plasma membrane; for others unknown enzyme systems; for Heilbrunn it is the protoplasmic colloids and the calcium ion. He certainly means to leave no doubt that nearly all uncertain cellular phenomena can be explained by changes in colloidal properties of protoplasm. Such an all-inclusive theory meets difficulties, as, for example, in the discussions of membrane phenomena. The evidence adduced by Schmitt, Danielli, and others for the molecular configuration of the plasma membrane is omitted, as is the role of polar-nonpolar structure of penetrating molecules. The role of ion exchange is scarcely mentioned, but accumulation of particular elements is accounted for by fixation on colloids in the cell interior.

An attempt is also made to account for the conduction of action potentials on a hypothesis of colloidal properties and loss of calcium from the cell cortex. It is stated that there is no convincing evidence for increase in permeability during conduction, but how else can the fall in electrical resistance be explained? The electrical parameters of the cell membrane described by Cole and others are omitted. Acetylcholine, sympathin, and histamine are discussed in detail, while the equally important part of potassium in nerve conduction is not included. Few nerve physiologists will admit the plausibility of the table (p. 535) purporting to show a graded change in calcium in nerve from the stimulated to the distal end. Heilbrunn's thesis is that the traditional Bernstein-Lillie model of a polarized membrane is inadequate to account for the known conduction phenomena. This is certainly true, but it is equally true that these phenomena cannot be accounted for in terms of colloids in the interior of the cell.

This book will be used by students as a textbook and by research biologists as a reference source. As a text there is much to recommend it—style, broad coverage of the field, and avoidance of excessive chemistry and physics. Some chapters might profitably be combined and repetition thus avoided, and more illustrations and descriptions of experimental methods would be helpful. Practically no new figures have been added in this edition. As a reference book the many citations and the summaries of a wide range of fields make the book very useful either for starting on the literature or for "brushing up" on a given topic. It is to be hoped that more biologists will become aware of the interesting field of general physiology because of this book.

PLANTS AND VITAMINS,[1] BY W. H. SCHOPFER; TRANSLATED BY N. L. NOECKER

Reviewed by G. W. Beadle
Stanford University

Although evidence for the existence of vitamins in plants was obtained early in the century, it was in animals under the impetus of medicine that really rapid strides were first made in vitamin research. Only within recent years has the general biological significance of these substances come to be widely appreciated. In elucidating this larger role, Schopfer has played a leading part. It is fitting, therefore, that he should be the one to bring together in *Plants and Vitamins* the information available from the plant side. In doing this, he has consolidated a vast amount of material not previously available in so convenient a form. He views this material through the eyes of a true biologist, recognizing that, with respect to the basic functions with which vitamins are concerned, plants and animals differ remarkably little.

With the wealth of material available and with the author's breadth of vision, one has the feeling that the stage was properly set for a classic. In the opinion of the reviewer, however, *Plants and Vitamins* falls short of this. Its organization and coherence are more nearly those of an excellent compilation than those of a great synthesis. Without doubt a higher order of unity would have been attained had not this book been written amid the turmoil of war.

Plants and Vitamins is divided into three parts: (I) synthesis of vitamins in plants; (II) vitamins in relation to plants unable to synthesize them; and (III) general problems involving vitamins. The title of the first part is perhaps somewhat misleading in suggesting to the uninitiated that he might find out *how* the plant synthesizes vitamins. Actually the reader finds out *which* vitamins are synthesized, *where* in the plant this is accomplished, and the effects of vitamin supplements. In chapters viii and ix it is revealed that, in fact, practically nothing is known about *how* vitamins are synthesized by plants. This gap in our knowledge is not, of course, limited to vitamins. Biochemists are well aware that very little is known about *how* organisms synthesize most substances of biological importance. Since obviously biosyntheses can be studied only in organisms in which they occur, it is evident that future advances in our knowledge of the mechanisms of the biosynthesis of vitamins will be made in studies of plants.

Part II includes discussions of the specific vitamin requirements of various groups of organisms as well as those of particular species. Chapter xiii deals with the functions of vitamins as coenzymes and constitutes a fitting tribute to the ingenuity and patience of the cellular physiologists who have solved many of the problems of how vitamins fit into respiratory enzyme systems. A chapter that will be of particular interest to the general biologist is one entitled "The Capacity for Synthesis and the Effect of Its Loss on the Organism." Here will be found discussed what might well be called the "Lwoff-Knight principle" of physiological specialization through progressive loss of synthetic ability. The implications of this principle and its genetic basis, so far as this is known, are indicated.

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[1] Waltham, Mass.: Chronica Botanica Co.; New York: G. E. Stechert & Co., 1943. Pp. xiv+293+3 pls. $4.75.

The final part of the book deals with such subjects as vitamins in nature, their importance in agriculture, vitamins as sex hormones in plants, ecological importance of vitamins in symbiosis and parasitism, and, finally, microörganisms as test objects for vitamins.

As a technical monograph, *Plants and Vitamins* will be found indispensable to those with an immediate interest in vitamins. In addition, it can be enthusiastically recommended to every biologist and biochemist. Even a casual reading of it cannot help but make one appreciate more fully the unity of living organisms.